UNIVERSIDADE FEDERAL DE MINAS GERAIS
Escola de Veterinária
Pós-graduação em Zootecnia

José Fernando Paz Ramírez

EFEITOS DO SISTEMA DE BIOFLOCOS E DA TEMPERATURA NA REDUÇÃO DA METILTESTOSTERONA DURANTE A MASCULINIZAÇÃO DA TILÁPIA DO NILO (*Oreochromis niloticus*)

Belo Horizonte
2025

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Tese apresentada ao Programa de Pós-Graduação em Zootecnia da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito parcial para obtenção do grau de Doutor em Zootecnia

Área de concentração: Produção Animal

Orientador: Prof. Dr. Eduardo Maldonado Turra

Co- orientadora: Dra. Érika Ramos Alvarenga

Belo Horizonte

2025

Ramírez, José Fernando Paz, 1983 -

R173e Efeitos do Sistema de Bioflocos e da Temperatura na redução da metiltestosterona durante a masculinização da Tilápia do Nilo (*Oreochromis niloticus*) / José Fernando Ramírez.- 2025.

116 f: il.

Orientador: Eduardo Maldonado Turra

Coorientadora: Érika Ramos Alvarenga

Tese (Doutorado) apresentado à Faculdade de Medicina Veterinária da UFMG, como requisito parcial para obtenção do título de Doutor em Zootecnia.

Área de Concentração: Produção Animal.

Inclui Bibliografia

1. Tilápia do Nilo - Peixe - Teses - 2. Aquicultura - Teses - I. Turra, Eduardo Maldonado - II. Alvarenga, Érika Ramos – III. Universidade Federal de Minas Gerais, Escola de Veterinária - IV. Título.

CDD – 639.3

Bibliotecária responsável Cristiane Patrícia Gomes CRB 2569

Biblioteca da Escola de Veterinária, UFMG.

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ATA DE DEFESA DE TESE DA ALUNO JOSÉ FERNANDO PAZ RAMIREZ

Às 08:30 horas do dia 22 de abril de 2025, reuniu-se, a Comissão Examinadora de Tese, aprovada em reunião ordinária no dia 11/04/2025, para julgar, em exame final, a defesa da tese intitulada: "Efeitos do sistema de bioflocos e da temperatura na redução da metiltestosterona durante a masculinização da tilápia do Nilo (*Oreochromis niloticus*)", como requisito final para a obtenção do Grau de **Doutor em Zootecnia, área de concentração Produção Animal.**

Abrindo a sessão, o Presidente da Comissão, Prof. Dr. **Eduardo Maldonado Turra**, após dar a conhecer aos presentes o teor das Normas Regulamentares da Defesa de Tese, passou a palavra ao (a) candidato (a), para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa do candidato (a). Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento da tese, tendo sido atribuídas as seguintes indicações:

	Aprovado	Reprovado
Prof.(a)/Dr.(a) Eduardo Maldonado Turra	X	
Prof.(a)/Dr.(a) Érika Ramos de Alvarenga	X	
Prof.(a)/Dr.(a) Edgar de Alencar Teixeira	X	
Prof.(a)/Dr.(a) Franklin Fernando Batista da Costa	X	
Prof.(a)/Dr.(a) Vinícius Monteiro Bezerra	X	

Pelas indicações, o (a) candidato (a) foi considerado (a):

[X] Aprovado (a)

[ ] Reprovado (a)

Para concluir o Doutorado, o(a) candidato(a) deverá entregar 03 volumes encadernados da versão final da tese acatando, se houver, as modificações sugeridas pela banca, e a comprovação de submissão de pelo menos um artigo científico em periódico recomendado pelo Colegiado dos Cursos. Para tanto terá o prazo máximo de 60 dias a contar da data defesa.

O resultado final, foi comunicado publicamente ao (a) candidato (a) pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora e encaminhada juntamente com um exemplar da tese apresentada para defesa.

Belo Horizonte, 22 de abril de 2025.

Assinatura dos membros da banca:

Eduardo M. Turra — Vinícius M. Bezerra

Érika Ramos de Alvarenga — [illegible]

Franklin F. B. Costa

Dedico à minha família, em especial à minha mãe, e a todos que, de alguma forma, deixam e levam algum conhecimento nessa eterna troca de aprendizados chamada vida. E a todos os que acompanharam e dividiram os momentos de doutorado. Minha gratidão!

AGRADECIMENTOS

Agradeço, principalmente, à minha mãe, Vera Lúcia Paz por seu amor, incentivo e por seu exemplo de força, persistência e sabedoria. Ao meu pai, José Ramírez, quem me deu uma profissão, e, acima de tudo me emprestou sua determinação, disciplina e paixão pelo conhecimento. À toda minha família, irmãs, primo, primas, tios, e em especial tia Beth Paz, por sempre acompanhar e fazer parte da minha trajetória, dando forças e incentivando. A meu afilhado Bernardinho, e seus pais Eduardo e Laura, por todo apoio e vibração com essa conquista.

A toda equipe NGT- Laqua, pelo aprendizado, troca diária e parceria que tivemos no dia a dia e em tantas coletas, manejos e reproduções. Aos colegas de pós Williane, Caroline, Bruno, Dara, Mariana, Laryssa e Vinícius por compartilharem momentos e aprendizados, e, em especial ao Franklin, por inúmeros conhecimentos que me foram transmitidos com sua experiência e por essa parceria que sempre dá certo, meu amigo. Agradeço a todos os IC's que trabalhamos juntos, dos mais antigos, Hugo, Isabela, Karen e outros, aos mais recentes. Sou grato, principalmente, àqueles que tiveram participação direta e importante nos experimentos aqui apresentados, como Kelly Keller, Gabriela Biscoto, toda equipe do ICB, Mariana, Franklin, Ana Paula, William, Natan, Ito e Lee. Sem a ajuda e participação de vocês, nada disso seria possível. Obrigado! Aos amigos Rafael e Vitor por tantos almoços no RU com várias conversas agradáveis e produtivas.

Agradeço aos professores do programa de doutorado em Zootecnia da UFMG, que contribuíram com conhecimentos importantes para meu desenvolvimento profissional, principalmente aos professores Daniela e Edgar, com quem sempre troquei ideias. Aos técnicos do Laqua, Franklin, Gabriel, Suelen e William que foram sempre muito solícitos ao longo dessa caminhada, além de essenciais para que tudo progredisse da melhor maneira. À equipe da coordenação Esther e, principalmente, ao Marcelo, sempre resolvendo questões de forma prestativa. Aos colegas e amigos do LAMA, vizinhos de laboratório, que sempre convidavam para um café e até ajudavam quando necessário. A equipe da faxina e aos porteiros do Laqua, em especial ao Lucas e ao Roberto, que tiveram sempre próximos, com inúmeras conversas e auxiliando em atividades essenciais.

Ao meu orientador, Eduardo Maldonado Turra, não tenho palavras para lhe agradecer, tanto pelo acolhimento na equipe, por tantas oportunidades que me foram dadas desde o início e por incontáveis conhecimentos que recebi. Para mim, és uma referência não só nas áreas de melhoramento genético, sistemas de produção e outras, mas em aliar pesquisa científica de qualidade com aplicabilidade no campo, que no fim das contas é o que importa e o que o setor precisa. À minha coorientadora Érika Ramos de Alvarenga, sempre brilhante em suas colocações, com uma inteligência rara para a pesquisa, que mistura conhecimentos estatísticos, biológicos, químicos e de escrita científica. Obrigado por ter me ensinado tantas e tantas coisa, sempre com muita paciência e atenção, sou extremamente grato por isso. Aos dois, a minha profunda admiração pela persistência, ética e rigorosidade na produção de ciência de alto nível em meio a tantas dificuldades.

Meu muito obrigado aos meus amigos Andres, Wendy, Fernando, Olman, Thaisa, Ana Paula e Liliana, os quais tornaram essa jornada muito mais leve e divertida, seja nos jantares, passeios, jogos de futebol ou num simples cafezinho para colocar a conversa em dia. É muito bom poder contar com vocês em momentos bons e ruins, meus amigos! A minha amiga Letícia Freixo, por sempre acompanhar minha trajetória, não só acadêmica, com muito acolhimento e incentivo. Ao Thiago Bernardes por ter indicado a UFMG para eu realizar meu doutorado, foi a escolha mais acertada possível, meu amigo, muito grato por isso e por sua parceria de sempre.

Agradeço à FAPEMIG pelo apoio financeiro em relação aos projetos desenvolvidos e à bolsa.

Estou e sou muito grato a todos vocês que fizeram parte dessa conquista! Obrigado!

“O que vale na vida não é o ponto de partida e sim a caminhada. Caminhando e semeando, no fim, terás o que colher."

Cora Coralina

RESUMO

A larvicultura da tilápia do Nilo em sua maioria é realizada em viveiros com grande presença de alimento vivo, variações de temperatura ao longo do dia e do ano, e sobras de ração por dissipação na água. Como consequência, o processo de masculinização precisa ser feito com alta concentração do hormônio sintético 17α-metiltestosterona (MT) na dieta. A produção em sistemas fechados, como a tecnologia de bioflocos (BFT), permite controlar a temperatura da água, utiliza um menor volume de água e gera menos efluentes. O BFT é um sistema alternativo para o processo de masculinização de tilápias do Nilo em viveiro, que demanda trocas de água em grandes volumes, gerando efluentes que podem conter resíduos de hormônio que pode causar impactos ambientais. Devido ao caráter de sistema fechado do BFT, nesta tese hipotetizou-se que seriam necessárias concentrações de MT menores do que as comumente utilizadas em viveiros (60 mg • Kg^{-1} de ração) para se obter altas taxas de masculinização. O primeiro estudo desta tese (Capítulo II) mostrou que o uso de 30 mg de MT · Kg^{-1} de ração resultou em masculinização >99% de tilápias em sistema BFT com troca zero de água. O estudo verificou também que manter a mesma água no tanque após o término do tratamento hormonal na continuidade da engorda dos alevinos não resultaria em maiores taxas de machos quando comparado ao manejo de substituição total da água. Além disso, a MT não foi detectada na água após 12 horas da última alimentação com ração contendo hormônio. Como foi possível obter uma alta taxa de masculinização utilizando 30 mg de MT · Kg^{-1} de ração, um novo experimento foi proposto reduzindo ainda mais essa concentração. O segundo estudo da tese (Capítulo III) avaliou as taxas de masculinização de larvas de tilápia do Nilo em sistema BFT com troca zero de água, combinando uma redução ainda maior de MT com diferentes temperaturas (25 e 28 °C). Com isso, foi possível verificar se a combinação entre concentrações reduzidas de MT e temperatura elevada seria suficiente para obter taxas elevadas de masculinização, reduzindo ainda mais a dependência de MT e a possibilidades de impacto no meio ambiente. No tratamento com temperatura a 28 °C as larvas cresceram 2,7 vezes mais, no entanto, apenas a temperatura mais elevada não foi suficiente para aumentar a masculinização, sendo estatisticamente similares nos tratamentos controle (0 mg · Kg^{-1} de ração), com 68,6% e 64,9% de machos nas temperaturas de 25 °C e 28 °C, respectivamente. Os

tratamentos que receberam hormônio na ração não divergiram entre as temperaturas 25 °C e 28 °C, apresentando taxas de masculinização acima de 98,7% e 96,9%, respectivamente. Portanto, é viável utilizar uma concentração ainda menor (10 mg de MT · Kg^{-1} de ração) em um BFT com troca zero de água, em temperatura da água igual ou acima de 25 °C. Por outro lado, a temperatura mais baixa tornou o processo de masculinização ainda mais sustentável, pois os peixes necessitavam de menores quantidades de ração devido à desaceleração do crescimento e, portanto, havia menos entrada de MT no sistema. Esses resultados dão suporte para uma mudança de paradigma na sustentabilidade da produção de tilápia e se aproximam de um novo protocolo voltado à masculinização em BFT com troca zero de água. Nosso estudo tem um impacto direto no cenário global de produção de tilápia, oferecendo uma masculinização eficiente e ambientalmente sustentável, diminuindo a demanda de água e de hormônios no processo, e assim e evitando a possível liberação de resíduos hormonais no meio aquático natural.

Palavras-chave:, Sistema intensivo, bioflocos, tilapia, metiltestosterona, sustentabilidade ambiental, temperatura, masculinização.

ABSTRACT

Nile tilapia larviculture is mostly carried out in ponds with a large presence of live food, temperature variations throughout the day and year, and leftover feed dissipated in the water. Therefore, the masculinization process needs to be carried out with a high concentration of the synthetic hormone 17α-methyltestosterone (MT) in the diet. Production in closed aquaculture systems, such as biofloc technology (BFT), allows controlling water temperature, using a smaller volume of water, and generating less effluent. BFT is an alternative system for the masculinization process of Nile tilapia in ponds, which requires large-volume water changes, generating effluents that may contain hormone residues that can cause environmental impacts. Due to the closed-system nature of BFT, it was hypothesized that it would be necessary to contain lower MT than those commonly used in ponds (60 mg • Kg^{-1} of feed) to obtain high masculinization rates. The first study of this thesis (Chapter II) showed that the use of 30 mg of MT · Kg^{-1} of feed resulted in >99% masculinization of tilapia in a BFT system with zero water exchange. The study also verified that maintaining the same water in the tank after the end of the

hormonal treatment during the growth of the fry did not result in higher male rates when compared to the management of total water replacement. In addition, MT was not detected in the water 12 hours after the last feeding with hormone-containing feed. Since it was possible to obtain a high masculinization rate using 30 mg of MT · Kg^{-1} of feed, a new experiment was proposed by further reducing this concentration. The second study of the thesis (Chapter III) evaluated the masculinization rates of Nile tilapia larvae in a BFT system with zero water exchange, combining an even greater reduction of MT with different temperatures (25 and 28 °C). Thus, it was possible to verify whether the combination of reduced MT concentrations and high temperature would be sufficient to obtain high masculinization rates, further reducing MT dependence and the possibility of impact on the environment. In the treatment with a temperature of 28 °C, the larvae grew 2.7 times more; however, the higher temperature alone was not sufficient to increase masculinization, being statistically similar in the control treatments (0 mg · Kg^{-1} of feed), with 68.6% and 64.9% of males at temperatures of 25 °C and 28 °C, respectively. The treatments that received hormone in the feed did not differ between temperatures of 25 °C and 28 °C, presenting masculinization rates above 98.7% and 96.9%, respectively. Therefore, it is feasible to use an even lower concentration (10 mg of MT · Kg^{-1} of feed) in a BFT with zero water exchange, at a water temperature equal to or above 25 °C. On the other hand, the lower temperature made the masculinization process even more sustainable, as the fish required lower amounts of feed due to the slowed growth and, therefore, there was less MT input into the system. These results support a paradigm shift in the sustainability of tilapia production and move closer to a new protocol aimed at masculinization in BFT with zero water exchange. Our study has a direct impact on the global tilapia production scenario, offering efficient and environmentally sustainable masculinization, reducing the demand for water and hormones in the process, and thus avoiding the possible release of hormonal residues into the natural aquatic environment.

Keywords: Intensive system, bioflocs, tilapia, methyltestosterone, environmental sustainability, temperature, masculinization.

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CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico

ANOVA - *Analysis of Variance*

APHA - American Public Health Association

ANVISA - Agência Nacional de Vigilância Sanitária - *National Health Surveillance Agency*

11βOHA4 - 11β-hidroxiandrostenediona

BFT - do inglês, *Biofloc Technology*

C:N - Carbono: Nitrogênio

DND - *Knockdown* de *dead end*

ESD - do inglês, *determinação ambiental do sexo*

FAO - Organização das Nações Unidas para a Alimentação e a Agricultura

FAPEMIG - Fundação de Amparo à Pesquisa do Estado de Minas Gerais

GPRA - Group of Pesticide Residue Analysts

GSD - do inglês, *determinação genotípica do sexo*

HPLC - *High performance liquid chromatography*

ICH - International Conference on Harmonization

IFREMER-COP - Instituto Francês de Investigação para a Exploração do Mar, Centro Oceanógrafo do Pacífico

LAQUA - Laboratório de Aquacultura

MT- *Metiltestosterona*

ME2 - *Methandrostenolone*

NGTAqua - Nutrição, Genética e Tecnologia em Aquacultura

RAS - do inglês, *recirculation aquaculture system*

SNK - *Student–Newman–Keuls test*

SS - *Settleable Solid*

TAN - *Total Ammonia Nitrogen*

N - *Total nitrogen*

TSS - *Total suspended solids*

UFMG - Universidade Federal de Minas Gerais

Sumário

1. CAPÍTULO I- APRESENTAÇÃO DA TESE

1.1 Introdução

A aquicultura é representada por todos os organismos aquáticos produzidos com finalidade comercial (algas, peixes, crustáceos, quelônios, crocodilianos, dentre outros). O relatório da Organização das Nações Unidas para a Alimentação e a Agricultura (FAO, 2024) para a produção aquícola mundial prevê uma produção de 205 milhões de toneladas para o ano 2032, o que corresponde a um aumento de 10%. O Brasil ocupa a décima segunda posição no ranking da produção aquícola, atrás de países do sudeste asiático, notavelmente menores, como Indonésia, Vietnã e Tailândia, que ocupam a 3ª, 4ª, 11ª posições, respectivamente, ou mesmo países da América, como Chile (8ª posição) e Equador (10ª posição) (FAO, 2024). Contudo, para a produção específica de tilápias, o segundo peixe mais produzido no mundo, atrás apenas das carpas, o Brasil é o quarto maior produtor mundial (FAO, 2024).

A tilápia é um ciclídeo, pertencente a ordem Cichliforme, que começou a despontar na aquicultura mundial a partir da década de 70 do século XX, majoritariamente, com doze espécies, distribuídas entre os gêneros *Oreochromis*, *Sarotherodon* e *Tilapia* (Baroiller e D'Cotta, 2018). As principais espécies produzidas atualmente na aquicultura pertencem ao primeiro gênero citado, e são: tilápia do Nilo (*Oreochromis niloticus*), mossâmbica (*Oreochromis mossambicus*), azul (*Oreochromis aureus*) e hornorum (*Oreochromis hornorum*). A espécie tilápia do Nilo tem destaque no cenário mundial e nacional devido ao rápido crescimento, fácil domesticação, baixa conversão alimentar, alta prolificidade, adaptabilidade alta em relação a diferentes tipos de parâmetros da qualidade da água e resistência a doenças (Baroiller e Toguyeni, 2004; Bezault et al., 2011).

A precocidade sexual da tilápia do Nilo é interessante para programas de melhoramento genético, como o desenvolvido pelo grupo de pesquisa NGTAqua (Nutrição, Genética e Tecnologia em Aquicultura) da Universidade Federal de Minas Gerais (UFMG), que seleciona tilápia produzida em bioflocos com base no ganho de massa num determinado período (Cavatti Neto et al., 2023). No entanto, esta precocidade

é indesejada para a produção, uma vez que se perde o controle reprodutivo e populacional, gerando lotes desuniformes, excesso de despescas que podem levar ao aparecimento de doenças, e manejo alimentar incorreto. Para contornar essa característica inconveniente para a produção é comum a utilização de populações monosexo nas tilapiculturas (Baroiller e D'Cotta, 2018). A produção de população monosexo na tilapicultura é vantajosa devido ao aumento da uniformidade nos lotes, crescimento mais acelerado, gasto energético direcionado para crescimento e não para desenvolvimento gonadal, melhor qualidade da carne (Hines e Watts, 1995; Beardmore et al., 2001; El-Sayed, 2006; Singh, 2013, Wang e Chen, 2018) e redução do estresse por competição e hierarquia, entre outros.

Dos métodos disponíveis para se obter uma população monosexo, o uso de hormônio masculinizante na ração é o mais utilizado, devido ao seu baixo custo e fácil aplicação (Baroiller e D'Cotta, 2018). O hormônio mais comum neste processo é a metiltestosterona (MT), devido a sua alta taxa de inversão para indivíduos machos.

O uso de hormônio na ração para masculinização de tilápia não gera pressões por parte dos mercados ou consumidores em relação a uma possível contaminação da carne, pois a quantidade de hormônio usado é muito pequena e há comprovações científicas mostrando que a MT é excretada após a fase de tratamento com hormônio, que dura 28 dias, e não se configura em um risco para a saúde humana. Além disso, o período de oferta de ração com hormônio é muito pequeno quando comparado ao período em que o peixe recebe ração sem hormônio, superior a cinco meses (Avnimelech et al., 2009, Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022).

Contudo, a masculinização costuma ocorrer em hapas instalados em viveiros, que demandam troca de água diária, e onde parte da ração com MT é perdida, resultando em efluentes contendo hormônio sendo liberados no meio ambiente (Murray et al., 2017). Ou seja, a MT pode ser liberada para os corpos d'água a cada troca de água, podendo se acumular no ambiente, bioacumular em alguns organismos e gerar uma magnificação trófica ao longo da cadeia alimentar, trazendo problemas ambientais. Outra preocupação é em relação a organismos que podem estar presentes no próprio viveiro, fora dos hapas, como peixes que escaparam, girinos ou outras espécies (Lone e Ridha, 1993; Homklin et al., 2011; Mlalila et al., 2015; Baroiller e D'Cotta, 2018). Realizar a masculinização em

sistemas fechados, como o sistema de recirculação de água ou o sistema de bioflocos, é uma tentativa sustentável que vem sendo explorada com o intuito de diminuir os possíveis impactos ambientais. Nestes, há a possibilidade de tratar a água usada durante a administração do hormônio de forma integral, sem descartes para o meio ambiente.

Embora não se tenha, até o momento, protocolos estabelecidos em relação às densidades ideais, concentrações adequadas de MT ou melhor temperatura para se fazer a masculinização em sistemas fechados, algumas pesquisas buscam elucidar essas questões. Assim, alguns trabalhos já compararam a masculinização com uso de MT na ração em sistemas de recirculação e no sistema de bioflocos (David-Ruales et al., 2019), assim como já foram testadas concentrações maiores do que a comumente usada em viveiros (Costa e Silva et al., 2022). O que se tem observado é que, provavelmente, seria possível reduzir a concentração de MT na masculinização realizada em sistema de bioflocos obtendo-se taxas de inversão similares às utilizadas em viveiros. Outro fator que ainda se conhece pouco em relação à masculinização no BFT é o papel da temperatura. Seria possível, utilizando uma combinação de temperaturas elevadas com pequenas concentrações de MT obter taxas de masculinização elevadas?

A temperatura afeta diretamente o ambiente e as taxas metabólicas dos organismos aquáticos (Rubalcaba, 2020), ou seja, interfere tanto nos parâmetros hídricos quanto no bem-estar, consumo e desempenho dos peixes. Dentre os fatores abióticos que podem interferir na diferenciação gonadal, a temperatura é o que mais se destaca (Baroiller e D'Cotta, 2001). Fora da faixa de temperatura considerada ótima, os peixes podem vivenciar uma situação estressante, levando a disfunções fisiológicas que podem prejudicar o desempenho e até levar à morte (Pörtner et al., 2017; Yang et al., 2021). Em temperaturas acima do limite ideal, pode ocorrer uma redução no consumo e no desempenho de crescimento dos peixes (Islam et al., 2020, Khieokhajonkhet et al., 2022). No entanto, temperaturas mais elevadas, dentro da faixa ideal para cada espécie, costumam estimular o consumo, otimizar a digestão e melhorar o desempenho do crescimento (Neuheimer et al., 2011; Volkoff e Rønnestad 2020).

1.2 Objetivos da tese

1.2.1 Objetivo Geral

O objetivo dessa tese foi verificar a possibilidade de reduzir a concentração de MT na masculinização de tilápia do Nilo no BFT com troca zero.

Avaliar os efeitos da temperatura no processo de masculinização de tilápia do Nilo no BFT com troca zero.

1.2.2 Objetivos Específicos

Realizar análises de qualidade da água para verificar possíveis influências da variação da concentração de MT e da temperatura na masculinização de tilápia do Nilo no BFT com troca zero.

Realizar avaliação do desempenho zootécnico para verificar possíveis influências da variação da concentração de MT e da temperatura na masculinização de tilápia do Nilo no BFT com troca zero.

Realizar a análise de aceto-carmim para proporção de machos.

Realizar e validar a técnica de HPLC para detecção de possíveis resíduos de MT na água após o período de alimentação com MT.

2. REVISÃO DE LITERATURA

2.1 Introdução

Dentre as características positivas da tilápia para produção em larga escala há a rusticidade, fácil reprodução, aceitação no mercado, e adaptação a diferentes sistemas produtivos. No entanto, a precocidade sexual da tilápia, que pode ocorrer aos 4 a 6 meses em condições adequadas de produção, não é interessante para a engorda (Baroiller e Toguyeni, 2004; Baroiller e D'Cotta, 2018), e, no caso da tilápia, o macho cresce mais do que as fêmeas, sem contar com o gasto energético e a privação de alimento durante o comportamento de proteção dos ovos na boca. A precocidade acarreta a reprodução

indesejada, levando a uma superpopulação, perda de controle sobre a densidade e oferta de ração, além de demandar despescas mais frequentes em função das diferenças faixas de peso corporal. Além disso, o estradiol, hormônio presente em maiores concentrações nas fêmeas, tem efeito inibitório no crescimento (Baroiller et al., 2014). Devido a todas essas características, os machos crescem mais e opta-se por produzir populações exclusivamente desse sexo (Wang et al., 2019; Teng et al., 2020).

Há algumas situações em que a produção de população mista, composta de machos e fêmeas, pode ser interessante. Para esse tipo de configuração produtiva usa-se o policultivo com predadores de ovos e larvas, ou o cultivo em altas densidade para controle da reprodução (Guerrero, 1982; Mair e Little, 1991). Contudo, o policultivo ou o adensamento não garantem que a reprodução não ocorra e cause problemas oriundos da superpopulação, como a competição por alimento e consequente limitação de crescimento (D'Amato, 2007). Esses tipos de estratégias, portanto, parecem ser viáveis para se produzir em sistemas pouco intensivos e com peixes de diferentes tamanhos, geralmente, pequenos e médios, o que atenderia as demandas africana ou asiática, que consomem peixes menores, com menos de 200 g (Little e Edwards, 2004).

No caso da produção que visa o mercado internacional, onde a demanda é por peixes maiores, existem outras estratégias para que os problemas causados pela precocidade não ocorram (Phelps e Popma, 2000). Dentre elas, há a hibridação e seleção (El-Sayed, 2006; Baroiller e D'Cotta, 2018; Wang Shen, 2018), a formação de triploides (Arai e Fujimoto, 2018; Alvarenga et al., 2020); e a produção de indivíduos monosexo machos, que pode ser obtida pela produção de supermachos (YY), que ao serem cruzados com fêmeas (XX) geram lotes formados por machos (XY) (Mair et al., 1995; Beardmore et al., 2001), por meio de tratamentos térmicos (Abucay et al., 1999; Pandit et al., 2015; Nozu e Nakamura, 2020), da realização de banho de imersão em água contendo hormônio, ou, via aplicação de hormônio na ração (Wassermann e Afonso, 2003; El-Sayed, 2006; Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022; Costa et al., 2024; Ramírez et al., 2024), e das técnicas de *Knockdown* de *dead end* (dnd) (fator essencial para as células germinativas) (Sawamura et al., 2017) e edição gênica CRISP/ Cas9 perspectiva de depleção de células germinativas (Liu, et al., 2015; Pradhan e Olsson, 2018).

Embora haja técnicas elaboradas e criativas entorno da tentativa de controle reprodutivo, a maioria carrega consigo algum tipo de limitação para que seja adotada em larga escala por tilapicultores. Na produção de machos XY, embora seja uma técnica considerada ambientalmente mais correta que as demais, possui o tempo como maior inconveniente, pois para produzir supermachos demanda uma série de testes de progênie e cruzamentos que podem levar vários anos (Baroiller e D'Cotta, 2018). Já no caso dos tratamentos térmicos, é comum que ocorra uma alta mortalidade, devido ao uso de temperaturas elevadas (Borges et al., 2005; Dias-Koberstein et al., 2006), além de poder elevar os custos com o uso de aquecedores, dependendo da região e época do ano, e de trazer à tona questionamentos em relação ao bem-estar animal. Já nas técnicas de *knockdown* e *knockout* existe a necessidade de número grande de ovos, tendo como risco mutações não desejadas em outros genes, tornando a técnica inviável até o presente momento (Liu, et al., 2015; Sawamura et al., 2017; Pradhan e Olsson, 2018). No caso do banho por imersão em água com hormônio, a taxa de masculinização costuma ser baixa (Phelps e Popma, 2000; Dias-Koberstein et al., 2006). E, por último, o uso de hormônio na ração, pode causar impactos ambientais, no entanto, é considerada a técnica mais eficaz e é a mais utilizada por produtores de tilápia.

2.2 Diferenciação sexual em tilápias

Existe uma grande variabilidade e plasticidade na determinação sexual dos peixes teleósteos (Devlin e Nagahama, 2002; Mank e Avise, 2009), que pode ser classificada como determinação genotípica do sexo (GSD, do inglês) e determinação ambiental do sexo (ESD, do inglês). Os fatores ambientais envolvidos neste processo podem ser fotoperíodo, pH, salinidade, densidade populacional e temperatura da água, dentre outros (Yao et al., 2021).

Sabe-se que existe um período, chamado de lábil, onde as gônadas podem seguir o caminho da diferenciação em testículos ou ovários, ainda que, genotipicamente, seu sexo seja o oposto. Neste período as células germinativas primordiais são indiferenciadas (Fostier et al., 1983; Piferrer, 2001) até que haja um estímulo hormonal, determinando o

sexo. Sendo assim, o período lábil coincide com ou inclui a diferenciação sexual fisiológica, momento pelo qual se tem maior sensibilidade aos esteroides sexuais exógenos (Fig 1.).

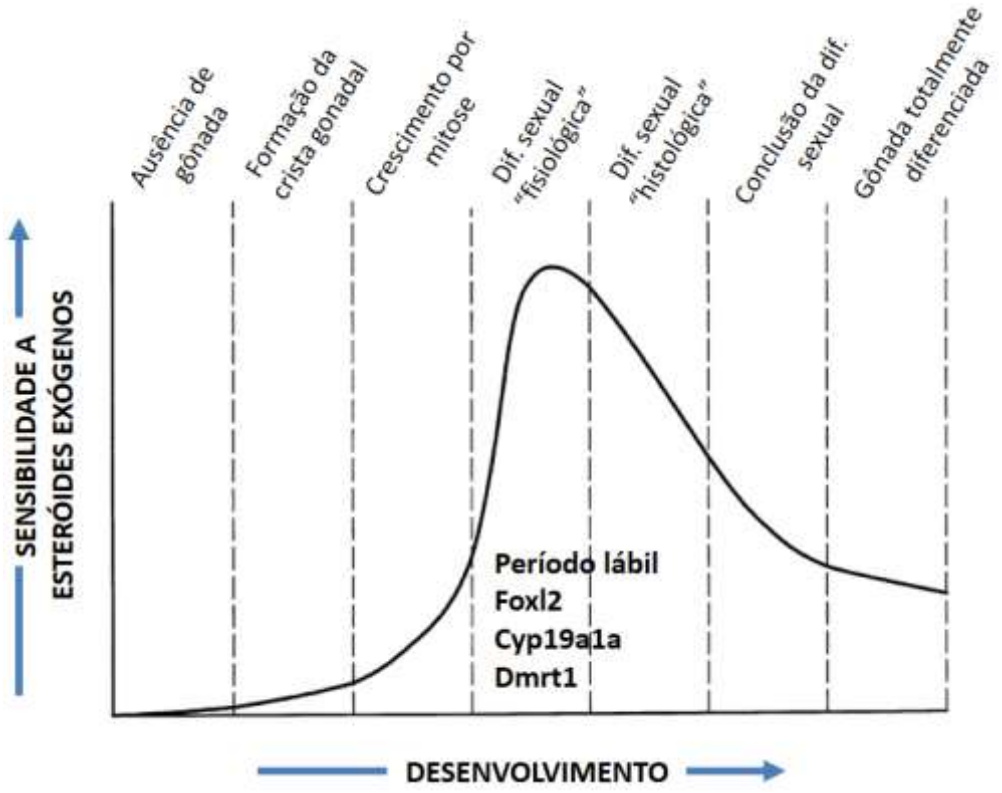

Figura 1. Mudanças de sensibilidade à ação de esteroides sexuais exógenos em relação aos eventos de gonadogênese, período lábil e expressão dos genes *Foxl2, Cyp19a1a* e *Dmrt1.* Adaptado de Piferrer (2001).

A diferenciação gonadal fica a cargo da enzima citocromo P450- aromatase, que catalisa a conversão de testosterona em 17-β-estradiol, hormônio que atua na diferenciação e desenvolvimento dos ovários (Li et al., 2014). Desta forma, pode haver fêmeas genotípicas desenvolvendo testículos devido a baixas concentrações de aromatase, e de forma contrária, pode haver machos genotípicos desenvolvendo ovários graças a níveis altos de aromatase. (Baroiller e D'Cotta, 2018).

Alguns genes estão envolvidos na diferenciação sexual, como relatado por Ijiri et al. (2008), que identificaram em tilápias do Nilo a partir de 5 dias após eclosão, a expressão dos genes Foxl2 e Cyp19a1a em gônadas XX, indicando um importante papel na diferenciação ovariana, enquanto os genes Dmrt1 apresentaram uma expressão masculina nas gônadas XY a partir de 6 dias após a eclosão, sugerindo interferir na diferenciação testicular, demonstrando que a expressão diferencial de genes que atuam em gônadas XX

e XY serão um fator crítico no período a partir de 5 a 6 dias após eclosão, determinando a diferenciação em ovários ou testículos de células indiferenciadas nesta espécie.

A produção de tilápias utiliza essa janela temporal de diferenciação sexual para formar animais com fenótipo masculino mesmo que contenham um genótipo XX de fêmeas. Com isso, a partir do uso de hormônios masculinos, produtores de tilápias ao redor do mundo utilizam hormônios esteroides masculinizantes para garantir o controle reprodutivo e uma produção monosexo (Hunter e Donaldson, 1983; Popma e Lovshin, 1996).

2.3 Masculinização em tilápia

Uma forma comumente usada em produções de tilápia para obtenção de população monosexo é a inversão sexual, que ocorre devido à versatilidade da diferenciação gonadal, e pode acontecer em função do uso de esteroides ou de fatores abióticos. Dentre os fatores ambientais, geralmente, a temperatura tem a maior influência na diferenciação gonadal e determinação sexual dos peixes (Baroiller e D' Cotta, 2001; Yao et al., 2021).

Assim como acontece para outras espécies, incluindo a tilápia, as altas temperaturas geram maior proporção de indivíduos machos, e para um número menor de peixes, baixas temperaturas estão associadas a maior quantidade de machos. Em trabalho com a tilápia *O. mossambicus* foram encontradas altas taxas de machos após tratamento com altas temperaturas, e taxa de fêmeas de, aproximadamente, 75% com peixes submetidos à 20 °C após 5 dias de eclosão, no entanto, utilizando baixas temperaturas após 10 dias de eclosão, não houve alteração da taxa de fêmeas (Wang e Tsai, 2000). Utilizando o robalo europeu *Dicentrarchus labrax* (Linnaeus, 1758), Pavlidis et al., (2000) encontraram alta proporção de fêmeas (70 a 73%) quando submeteram os peixes à baixas temperaturas (13 a 15 °C), 30 horas após a fertilização.

A explicação para a inversão sexual por temperatura em peixes reside na capacidade da enzima citocromo P450- aromatase catalisar a conversão de testosterona em 17-β-estradiol, hormônio que atua na diferenciação e desenvolvimento dos ovários. O tratamento com temperatura tem ação reguladora negativa na expressão do gene responsável por expressar essa enzima, ou seja, fazendo com que inicie um processo de desenvolvimento de estruturas sexuais masculinas associadas com o desenvolvimento de

testículos funcionais. Em larvas de tilápia submetidas à temperatura de 35 °C durante o período de diferenciação, ocorre masculinização de fêmeas XX devido ao bloqueio desta enzima (Guiguen et al., 1999; Baroiller e D'Cotta, 2001).

A elevação da temperatura tem sido apresentada como uma forma de inversão sexual, porém, altas temperaturas podem ser acompanhadas de alta taxa de mortalidade, como observaram Borges et al. (2005) submetendo larvas à temperatura de 37 °C por 28 dias e obtendo 69% de sobrevivência. Por outro lado, temperaturas mais baixas (34 °C) foram testadas por Azaza et al. (2008) sem que se obtivesse masculinização superior a 52%. Além disso, o uso de tratamento por temperatura pode aumentar os custos com energia com o uso de aquecedores para elevar a temperatura por um determinado tempo. Esses fatores citados acima, e a falta de respostas que gerem um padrão tornam o método da masculinização por temperatura uma realidade ainda não aplicável em produção comercial.

Assim como ocorre com a inversão por temperatura, o uso de tratamentos hormonais também deve ocorrer durante o período lábil, momento em que ocorre a maior sensibilidade para ser realizado esse processo, ou seja, antes e durante a fase de diferenciação sexual dos tecidos gonadais, como observado nas espécies tilápia (Baroiller e Toguyeni, 1996) e bagre do canal (*Ictalurus punctatus*) (Patino et al., 1996). Em tilápia, a fase crítica de diferenciação das gônadas coincide com o período um pouco após os primeiros lançamentos das larvas para fora da boca da mãe, ou seja, entre 10 a 12 dias após a fertilização, em seguida do período de incubação e absorção do saco vitelínico (Baroiller e D'Cotta, 2001).

A utilização de hormônio por banho de imersão, que tem sido utilizado desde 1965 (Phelps e Popma, 2000), requer a exposição dos peixes a uma água contendo hormônio. É um método com menores custos, e pode ser realizado em estruturas pequenas, com maior controle sobre os efluentes. Segundo Bombardelli e Hayashi (2005), o crescimento e a sobrevivência de larvas não foram influenciados pela masculinização por banho de imersão. Entretanto, a taxa de masculinização por banhos de imersão tem se mostrado menos eficaz do que a aplicação de hormônio na ração (Phelps e Popma, 2000). Em trabalho realizado por Dias-Koberstein et al. (2006), foram utilizadas diferentes concentrações de MT (1, 3 e 6 $mg.L^{-1}$) para masculinização de tilápia do Nilo por banho

de imersão, tendo obtido a maior porcentagem de machos (84%) usando a concentração de 6 $mg.L^{-1}$.

A aplicação de hormônio masculino na ração é o método mais frequentemente usado na produção mundial de tilápia devido à sua praticidade e eficácia, que alcança em média 98% de indivíduos machos (Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022; Sarker et al., 2022). A taxa de inversão pode estar relacionada com o número de dias de oferta de ração com hormônio, o tipo de hormônio usado, o sistema de produção, a quantidade de ração ofertada por dia, além do armazenamento adequado da ração. A ingestão de MT em excesso, no entanto, pode ocasionar o que é conhecido como "efeito paradoxo da inversão sexual", em que ocorre o contrário da masculinização, formando indivíduos não machos (Guerrero, 1975; Cruz and Mair, 1994; Pandian and Sheela, 1995; Beardmore et al., 2001; El-Sayed, 2006).

O hormônio mais comumente utilizado na produção de tilápia é o 17-α-metiltestosterona (MT) devido ao seu baixo valor de mercado, fácil aplicação e alta confiabilidade em relação à taxa de masculinização (El-Sayed, 2006; Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022; Sarker et al., 2022). O fornecimento de ração com MT na água inicia no momento seguinte ao final do consumo do saco vitelínico, que deve ocorrer em larvas com, aproximadamente, 10 a 11 mg e por volta do décimo dia após a fertilização dos ovos (Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022). Alguns hormônios produzidos naturalmente na diferenciação testicular, como o 11β-hidroxiandrostenediona (11βOHA4), já foram testados em tilápia do Nilo e na tilápia vermelha utilizando 50 $mg.\ Kg^{-1}$ de ração, e alcançaram taxas de machos semelhantes às da MT (Desprez et al., 2003). Todavia, embora eficazes e de fácil eliminação pelos peixes, quando comparados a um hormônio artificial, os hormônios naturais não são muito utilizados na tilapicultura devido ao seu alto valor no mercado.

A MT é oferecida durante o período de tratamento hormonal, que dura, geralmente, de 28 a 30 dias a partir do consumo do saco vitelínico e é, com frequência, ofertado na concentração de 60 mg $MT.Kg^{-1}$ de ração. No entanto, essa concentração é usada em viveiros, que possuem grande quantidade de fitoplâncton e microrganismos e onde há perda de ração (Baroiller e D'Cotta, 2018; Costa e Silva et al., 2022), além de grande incidência de radiação solar, que podem levar à fotólise direta, oxidação por UV

e isomerização. A possível eficácia do uso de menores concentrações de MT em sistemas fechados, como na recirculação e no BFT, para realização da masculinização já havia sido proposto por Baroiller e D'Cotta (2018) e Costa e Silva et al (2022).

De maneira geral, se utiliza 50 ml de etanol (95%) para dissolver 50-60 mg MT. Kg^{-1} de ração (Baroiller e D'Cotta, 2018), porém é comum encontrar essa mesma diluição em 200 ml de etanol (95%) (Costa e Silva et al., 2022, Costa et al., 2024). Devido ao caráter lipossolúvel da MT, também é possível usar o óleo de soja como veículo (Costa et al., 2024).

2.4 Possíveis impactos do uso de MT

Alguns estudos evidenciam que o hormônio MT utilizado para a masculinização de peixes não permanece na carcaça, apesar disso, existe uma preocupação ambiental em relação ao efluente resultante desse processo, que pode gerar questionamentos voltados para a segurança da alimentação humana (Lone e Ridha, 1993; Mlalila et al., 2015).

A defesa da ideia de que a MT não representa um risco para a saúde humana se fundamenta em três principais argumentos (Baroiller e D'Cotta, 2018; Thanasupsin et al., 2021). Um deles diz respeito ao período de aplicação e a quantidade de MT ofertada por larva. O uso de hormônio, comumente, ocorre durante um curto período, sendo iniciado logo na primeira alimentação até, no máximo, quatro semanas, e, corresponde a uma quantidade de apenas 0,047mg de MT por larva (Baroiller e D'Cotta, 2018). A outra argumentação a favor da segurança alimentar humana se refere à rápida degradação e excreção da MT pelos alevinos (Piferrer, 2001). E, por último, a questão do tempo restante de engorda, cinco meses ou mais, em que se oferta ração livre de hormônio, sendo tempo suficiente para a MT, que foi oferecida nos primeiros 28 dias, ter sido completamente eliminada do organismo dos peixes (Baroiller e D'Cotta, 2018).

Entretanto, a partir da MT podem ser formados metabólitos devido à biotransformação ocorrida no organismo dos peixes, e, ainda não se sabe, ao certo, quais vias bioquímicas são acionadas neste processo. Dentre os compostos que são gerados podem ser citados o 17alpha-methyl-5-xi-androstan-3xi, o 17alpha-methyl-4-androsten-

17beta-ol-3 e o 17alpha-methyl-17beta-hydroxy-4,6-androstadiene-3-one (Cravedi et al., 1993). O risco desses metabólitos se acumularem nos órgãos dos peixes e afetar seus consumidores é considerado inexistente devido à sua pequena quantidade por peixe, como ocorre em relação ao produto original (MT), e à sua rápida eliminação após o período de tratamento hormonal.

Com isso, o risco e a preocupação maior passam a ser em relação ao meio ambiente, pois estes metabólitos podem se acumular e não se tem informações suficientes sobre seus efeitos e impactos ambientais. Por sua natureza lipofílica, a MT não se dissolve na água e pode se aderir às partículas e matéria orgânica do solo do viveiro, e sua adsorção, acúmulo e descolamento nessas superfícies dependem de fatores como temperatura, pH, carga iônica, tamanho da partícula do solo, dentre outros (Qi e Zhang, 2016).

A masculinização de pós-larvas de tilápia, geralmente, ocorre em hapas instaladas em viveiros, que demandam renovação de água diária. Ou seja, o resíduo do hormônio, das fezes ou da ração não consumida, pode ficar presente na água da produção e ser descartado no meio ambiente. Mais ainda, peixes maiores, anfíbios e outros animais que podem ficar do lado de fora da hapa, no viveiro, podem consumir a mesma ração usada na masculinização e acumular MT no tecido adiposo (Homklin et al., 2011; Baroiller e D'Cotta, 2018). Até mesmo pássaros piscívoros podem se alimentar de larvas ou peixes maiores fora da hapa que ingeriram ração contendo hormônio, e não se sabe se isso pode causar alterações fisiológicas nestes e em outros animais predadores que, eventualmente, visitam os viveiros em busca de alimento, e podem bioacumular a MT (Murray et al., 2016; Murray et al., 2017).

Dessa forma, a masculinização em sistemas fechados, como o sistema de recirculação de água e o sistema de bioflocos, emergem como alternativas sustentáveis neste manejo, reduzindo os impactos ambientais, uma vez que permitem um maior controle da água e dos peixes. Sistemas com maior grau de intensificação permitem aumentar a produtividade sem a necessidade de aumentar o espaço ou o uso da água (Costa e Silva et al., 2022; Avnimelech et al., 2008).

2.5 Sistemas fechados: bioflocos e recirculação de água

A tecnologia de recirculação de água, conhecida, popularmente, pela sigla RAS, (do inglês, *recirculation aquaculture system*) pode ser usada na masculinização com uso de hormônio na ração, porém, comparado ao sistema de bioflocos (BFT), tem mais elementos que podem representar riscos durante seu funcionamento, como por exemplo a necessidade de filtros mecânicos e biológicos. Mais estruturas além dos tanques de peixes, assim como a dependência de uma bomba d'água, tornam o sistema de recirculação um investimento maior. O RAS, no entanto, é interessante em escalas menores, como na produção de formas jovens de peixes ornamentais (Avnimelech, 2006). Enquanto o RAS demanda estruturas de filtragens, além de uma bomba d'água, o sistema BFT possui como estrutura física apenas o tanque de peixe e uma fonte de aeração, ou seja, requer um menor investimento e, é mais simples de operar, mesmo em sistemas que utilizam clarificadores com pequeno bombeamento.

A tecnologia de produção aquícola em bioflocos, conhecida como "BFT" (do inglês, *Biofloc Technology*), "*aerated microbial reuse systems*", "*active suspension pond*", entre outras denominações (Hargreaves, 2006) surgiu no Instituto Francês de Investigação para a Exploração do Mar, Centro Oceanógrafo do Pacífico (IFREMER-COP), na década de 1970. Essa forma de produzir emergiu como uma alternativa sustentável para produção de organismos aquáticos (Avnimelech, 2009; Crab et al., 2012; Ahmad et al., 2017; Khanjani et al., 2022), tendo como diferencial a troca mínima ou "troca zero" de água , o uso de densidades maiores do que em viveiros (Naylor et al., 2000; Avnimelech, 2009; Crab et al., 2012; FAO, 2016; Khanjani et al., 2022) e o desenvolvimento de populações de bactérias capazes de transformar os resíduos dos peixes em uma biomassa de flocos nutritivos, também capazes de eliminar compostos que prejudicam a qualidade da água (Emerenciano et al., 2013; Ahmad et al., 2017).

Anos depois, a tecnologia de produção em BFT teve um impulsionamento na década de 1990, quando o Centro de Maricultura de Waddell, localizado nos Estados Unidos, produziu trabalhos que visavam o aumento de produtividade da carcinicultura

marinha com pequenas taxas de renovação de água, ao passo que Israel desenvolvia estudos com a produção de tilápia em sistema de bioflocos (Avnimelech, 2009).

Os flocos do BFT são formados por meio do estímulo da proliferação de bactérias que cumprem o papel de remover a amônia, um composto que pode ser tóxico para os peixes. A produção e manutenção do bioflocos consiste na adição de uma fonte de carbono orgânico para se chegar a uma proporção adequada em relação ao nitrogênio. Essa proporção, comumente chamada de relação C/N, permite a condição favorável para o desenvolvimento de bactérias chamadas heterotróficas. Contudo, no sistema de bioflocos, a remoção de amônia não fica a cargo única e exclusivamente destas bactérias, há também a formação de outro grupo, as bactérias quimioautotróficas, que desempenham a mesma função removedora de amônia na água, porém, de maneira diferente, e dependem da manutenção da alcalinidade para utilização de carbonatos e bicarbonatos como fonte de carbono (Avnimelech, 2006; Schneider et al., 2005; Schneider et al., 2006).

Os flocos além de conter as bactérias citadas anteriormente, possuem também protozoários, zooplânctons e, em determinadas condições de luminosidade, fitoplânctons, que podem estar presentes em maior ou menor quantidade em função da transparência da água. Essa massa, chamada de flocos, formada pela agregação desses microrganismos, assim como, por células mortas, coloides e polímeros orgânicos, não somente tem relevância na remoção de amônia, mas também serve de alimento para os animais filtradores, atuando como uma fonte de nutrientes (McIntosh, 2000; Burford et al., 2004; Wasielesky et al., 2006). A composição dos flocos pode ter entre 60 e 70% de parte orgânica, 30 a 40% de parte inorgânica e entre 2 e 20% de células vivas (Wilen et al., 2003). A presença dessa massa nutritiva pode, portanto, refletir em uma menor necessidade de ração e, consequentemente, na redução dos custos, principalmente na fase inicial.

O período de formação e maturação do bioflocos requer bastante atenção em relação aos parâmetros da qualidade da água. Devido à ausência de bactérias em volume suficiente para remover a amônia proveniente, principalmente, da excreção branquial e da mineralização da matéria orgânica, ocorrem picos desse composto assim como do nitrito (Hargreaves, 2013).

A migração de sistemas menos intensivos para sistemas mais intensivos contribuiu para o aumento de pesquisas relacionadas a produção de tilápia em BFT, tecnologia que vem se consolidando devido a características que podem contribuir para o desenvolvimento da cadeia produtiva desta espécie (Turra et al., 2016; Alvarenga et al., 2018; Manduca et al., 2020, Manduca et al., 2021, Costa e Silva et al., 2022). O maior controle da produção e dos parâmetros da água, o uso racional da água, a diminuição da quantidade e do custo com ração, a menor quantidade de estruturas, e o menor gasto com energia, assim como a possibilidade de tratamento dos resíduos, tornam o BFT um sistema cada vez mais interessante (Luo et al., 2014; Avnimelech, 2009). No entanto, ainda há muitas lacunas a serem preenchidas para que se possa consolidar padrões que tornem o sistema mais eficaz e, de fato, viável e sustentável do ponto de vista ambiental e econômico.

Utilizando duas estratégias de uso da água, Manduca et al. (2020) realizaram estudos com densidades diferentes. No primeiro experimento, peixes com aproximadamente 100g foram mantidos em densidades de 20; 40; 60 e 80 peixes/ m^3 até o peso de 250g, sem realizar troca de água, apenas reposição da água evaporada. Com modelos matemáticos chegou-se à densidade considerada ideal para essa estratégia de troca zero, de aproximadamente 52 peixes/ m^3, o que correspondeu a 13 Kg/m^3. No segundo experimento (Manduca et al., 2021), os peixes iniciaram com, aproximadamente, 130g e foram mantidos até o peso comercial de 700g, porém, neste foram realizadas pequenas trocas de água (equivalente a 5% ao dia), e a densidade ideal foi de 33 peixes/ m^3, o que representaria, aproximadamente, 23 Kg/m^3. A conclusão dos autores é que pequenas trocas impedem o acúmulo de nitrato e de sólidos suspensos em excesso, fatores que limitam a qualidade da água, prejudicando o desempenho zootécnico e a sobrevivência, e, que mesmo com essas trocas de água, altas densidades podem levar um número baixo de peixes a alcançar o peso de abate no tempo esperado.

O sistema de bioflocos também auxilia na redução de agentes patogênicos (Hargreaves, 2013), o que torna essa tecnologia interessante especialmente para a fase inicial, quando se realiza a masculinização, período sensível que ocorrem altas taxas de mortalidade e doenças. No entanto, ainda há poucos estudos voltados para o manejo da masculinização em sistema de bioflocos.

Comparando a masculinização realizada em RAS e em BFT, utilizando a dosagem de 60 mg MT.Kg^{-1} de ração, David-Ruales et al. (2019) concluíram que no BFT as larvas tiveram melhor desempenho zootécnico para as variáveis tamanho final, crescimento diário e fator de condição, no entanto, a taxa de masculinização foi superior no RAS (91% de machos) do que no BFT (61% de machos). Os autores atribuíram a redução da eficiência de reversão sexual no BFT à valores de sólidos sedimentáveis superiores a 35ml/ L, contribuindo com menor ingestão de ração, e, logo, de hormônio masculinizante. Cabe salientar, que a taxa de arraçoamento neste experimento foi de apenas 10% do peso vivo ao longo de todo o experimento, reduzindo assim, o consumo de ração, resultando em uma menor ingestão de hormônio, enquanto outros trabalhos sugerem iniciar com 30% na primeira semana e reduzir 5% a cada semana, até alcançar 15% na quarta semana (Costa e Silva et al., 2022; Do Valle et al., 2023; Costa et al., 2024). Iniciar a primeira semana de oferta de ração contendo MT com uma taxa de arraçoamento de 30% do peso vivo é importante pois coincide com o período de maior sensibilidade da tilápia do Nilo ao hormônio durante o período lábil, como mostrado na figura 1. A redução da taxa para até 15% do peso vivo é justificada pelo princípio de alometria proposto por Von Bertalanffy (1938), em que as taxas metabólicas e de crescimento são reduzidas ao longo do tempo, demandando menores taxas de alimentação à medida que os peixes crescem. Além disso, fazendo uma média das taxas usadas ao longo de 28 dias (30%, 25%, 20% e 15%) temos uma taxa de 22,5% do peso vivo ao longo de todo o experimento, valor que está de acordo com autores que utilizam entre 15% e 30% do peso vivo e obtiveram altas taxas de masculinização de tilápia com MT (Shepperd, 1984; Phelps et al., 1992; Green and Teichert-Coddington, 1994, Okoko, 1996; El-Greisy et al., 2012).

Estudo realizado por Costa e Silva et al. (2022) testou a masculinização de tilápia do Nilo em BFT usando concentrações maiores (60, 90, 120, 150 e 180 mg MT.Kg^{-1} de ração) que a concentração comumente usada em viveiros (60 mg MT.Kg^{-1} de ração). A hipótese desse estudo partiu do pressuposto que no BFT a ingestão de ração pelas larvas seria reduzida, uma vez que também se alimentariam de flocos com nutrientes, logo, necessitaria uma maior concentração de MT para alcançar taxas de masculinização tão altas quanto as alcançadas em viveiros. Porém, os resultados apontaram o contrário, os pesquisadores concluíram, por meio de análise de regressão, que quanto maior a

concentração de MT menor era a porcentagem de machos. Os grupos que receberam dosagens maiores de MT apresentaram uma quantidade de machos inferior ao grupo cuja dosagem foi de 60 mg MT.Kg^{-1} de ração (≥ 94% de machos), valor semelhante à taxa de masculinização em viveiros, indicando que no BFT essa mesma dosagem seria suficiente para alcançar resultados satisfatórios. Em decorrência desses resultados, alguns autores propuseram que fossem realizados estudos para testar a masculinização em concentrações menores no sistema BFT (Baroiller e D'Cotta, 2018; Costa e Silva, et al., 2022), com a possibilidade de se aumentar ainda mais sua sustentabilidade ambiental.

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3. CAPÍTULO II- REDUCTION OF METHYLTESTOSTERONE CONCENTRATION IN FEED DURING MASCULINIZATION OF NILE TILAPIA (O*reochromis niloticus*) IN BIOFLOC SYSTEM

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Published: Aquaculture 593:741253, June, 2024

Aquaculture 593 (2024) 741253

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Reduction of methyltestosterone concentration in feed during masculinization of Nile tilapia (*Oreochromis niloticus*) in biofloc system

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ARTICLE INFO

Keywords:
Biofloc
Intensive system
Nile tilapia
Methyltestosterone
Sustainability

ABSTRACT

Most larviculture of Nile tilapia is carried out in ponds with a large presence of live food, with temperature variations throughout the day and year, and feed leftovers due to dissipation in the water. As a consequence, the masculinization process needs to be done with a high concentration of 17α-methyltestosterone (MT) in the diet. Production in closed systems, such as biofloc technology (BFT), allows controlling the water temperature, uses a smaller volume of water and generates less effluent, being an alternative system for the masculinization process. This study evaluated the masculinization rates of Nile tilapia in the BFT, with a controlled temperature (~28 °C), using concentrations of MT in the feed lower than those recommended for masculinization in ponds and BFT (60 mg • Kg^{-1} of feed). Tilapia larvae (post yolk sac absorption) were raised in BFT (2 larvae • L^{-1} or 100 larvae • $tank^{-1}$) and submitted to different concentrations of MT in the diet (0, 30, 40, 50 and 60 mg • Kg^{-1} of feed), which was offered five times a day for a period of 28 days. The experimental design was entirely randomized. After the hormonal treatment, the water in the tanks was completely replaced with new water, free of hormonal residues, where the fry remained until they reached a viable size for sexing. It was also tested to maintain the original water used during the hormonal treatment period, during the post-experimental period, where 30 mg • Kg^{-1} of feed was used. The water quality variables (temperature, pH, dissolved oxygen, settleable solids, total suspended solids, alkalinity, nitrogen compounds, salinity, phosphate and organic carbon) and growth performance variables (final body weight and length, specific growth rate, condition factor and survival) did not differ between MT concentration in the diet ($p > .05$). The treatment without hormone (control) presented 73.6% of males and the treatments that received hormone showed masculinization rates > 99%, significantly higher than control ($p < .05$), but did not differ from each other ($p > .05$). The percentage of male individuals in the control treatment was high and this may have occurred because of the temperature of 28 °C throughout the experiment. However, this percentage of males is not considered high enough for production, which requires higher rates close to 100% as possible. The MT was not detected in the water 12 h after the last feeding with feed containing hormone. Thus, it is feasible to use a lower concentration (30 mg of MT • Kg^{-1} of feed) in BFT. Our study has a direct impact on the global tilapia production scenario, offering a new environmentally sustainable masculinization protocol, using less water and hormones in the process and avoiding the release of hormonal residues.

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https://doi.org/10.1016/j.aquaculture.2024.741253
Received 6 March 2024; Received in revised form 22 May 2024; Accepted 17 June 2024
Available online 18 June 2024

3.1 Abstract

The masculinization of Nile tilapia, through dietary hormonal treatment, is still an environmentally unfriendly process. Most larviculture is carried out in ponds, with the larvae stocked or not in hapas. As it is an open system, with a large presence of live food in the water, with many temperature variations throughout the day and year, and feed leftovers due to dissipation in the water, the masculinization process needs to be done with a high concentration of 17α-methyltestosterone (MT) hormone in the diet. Furthermore, water changes in large volumes are carried out in this production system, generating a large amount of effluents that may contain hormone residues and cause environmental impacts. Production in closed systems, such as biofloc technology (BFT), allows controlling the water temperature, uses a smaller volume of stored water and generates a smaller volume of effluents, being an alternative system for the masculinization process. This study evaluated the masculinization rates of Nile tilapia in the BFT system, with a controlled temperature of around 28 °C, using concentrations of MT in the feed lower than those commonly recommended for masculinization in ponds and also BFT (60 mg · Kg^{-1} of feed) with the possibility of reducing this impact on the environment. Nile tilapia larvae (post yolk sac absorption) were raised in BFT and submitted to different concentrations of 17α-methyltestosterone in the diet (0, 30, 40, 50 and 60 mg · Kg^{-1} of feed), which was offered five times a day for a period of 28 days. The experimental design was entirely randomized. Stocking density applied was of 2 larvae · L^{-1} (100 larvae · $tank^{-1}$). After the hormonal treatment period, the water in the tanks was completely replaced with new water, free of possible hormonal residues, where the fry remained for around 15 days, until they reached a viable size for sexing. It was also tested to maintain the original water used during the hormonal treatment period, during the post-experimental period, with 4 other tanks where 30 mg · Kg^{-1} of feed was used. The water quality and growth performance variables did not diverge between MT concentration levels in the diet ($p > .05$). The treatment without hormone in the diet (control with 0 mg · Kg^{-1} of feed) presented 73.6% of males and the treatments that received hormone in the feed showed masculinization rates greater than 99% and did not differ from each other ($p > .05$). The analysis of MT in the water, 12 hours after the last feeding with feed containing hormone, did not detect the presence of the hormone in the water. In

conclusion, it is feasible to use a lower concentration (30 mg of MT · Kg^{-1} of feed) in BFT and, in this way, reduce possible environmental impacts.

Key-words: bioflocs, intensive system, Nile tilapia, metiltestosterone, masculinization, sustainability.

3.2 Introduction

The sexual precocity of Nile tilapia is important for genetic improvement programs, which evaluates and selects based on body weight of Nile tilapias candidates produced in bioflocs (BFT), increasing genetic gain per year (Cavatti Neto et al., 2023). However, for fish farmers, who raise the tilapia for fish market, early sexual maturation is undesirable, as reproductive and population control is lost, leading to uneven batches, reduced growth, and incorrect feeding (Farahmand et al., 2007; Baroiller et al., 2014). To overcome these inconveniences, it is common to raise a monosex population in tilapiculture. Of the methods available to obtain monosex population, the masculinization process by masculinizing hormone in feed is the most used, due to its low cost, high efficiency, and easy application (Baroiller and D'Cotta, 2018; Costa e Silva et al., 2022). The most common hormone for this methodology is 17α-methyltestosterone (MT), due to its high rate of masculinization (Sarker et al., 2022).

The use of MT for tilapia masculinization raises few doubts regarding meat contamination, as there are several studies showing that MT residue is metabolized by fry and excreted after the hormone treatment phase (Piferrer, 2001). In a study carried out by Rothbard et al. (1990), it was demonstrated that after 11 weeks of hormonal treatment of tilapia with 60 mg of 17α-ethynyl testosterone per kg of feed, the hormone concentration obtained in fish muscle was 168.6 ng · g^{-1} and 142.1 ng · g^{-1} at the first- and second-days post hormone treatment, respectively. Samples collected on days 3, 5 and 7 did not differ from the control, not treated with 17α-ethynyl testosterone, and were below the detectable level (50 ng · g^{-1}). In another study, Curtis et al. (1991) offered 30 mg · Kg^{-1} of 17α-methyltestosterone over 30 days and there was a logarithmic reduction in hormone concentration between the first and tenth day after treatment. Therefore, the consumption of this fish in adulthood does not pose a risk to human health (Baroiller and D'Cotta, 2018; Thanasupsin et al., 2021).

The biggest concern regarding the use of MT in tilapiculture, therefore, is related to environmental contamination, as there is still not enough information about how its degradation occurs (Lone and Ridha, 1993; Mlalila et al., 2015). It is known that metabolites can be formed from MT due to biotransformation occurring in the fish organism or in the environment itself, however, it is still unclear which biochemical pathways are activated in this process. In the liver, hormones are metabolized and form

water-soluble compounds, being released within hours or days after treatment, through bile and urine excretion (Specker and Chandlee, 2003). Among the compounds that are generated, 17alpha-methyl-5-xi-androstan-3xi, 17alpha-methyl-4-androsten-17beta-ol-3 and 17alpha-methyl-17beta-hydroxy-4,6- androstadiene-3-one (Cravedi et al., 1993).

Tilapia masculinization is commonly carried out in the pond production system (with the use of hapas or not), which often requires daily water changes. The result of this practice is the release of effluents containing hormone residues into the environment (Murray et al., 2017). Due to its lipophilic nature, non-ingested MT dissolves in water and can adhere to particles and organic matter in the pond soil, and its adsorption, accumulation and displacement on these surfaces depend on factors such as temperature, pH, ionic charge, particle size soil, among others (Qi and Zhang, 2016). In other words, MT residues, originating from unconsumed feed or fish excretion, can remain present in the pond and be discarded into the environment with each water change, potentially bioaccumulating in some organisms and generating trophic magnification along the food chain (Homklin et al., 2011; Baroiller and D'Cotta, 2018).

Another concern is in relation to organisms that may be present in the pond itself, outside the hapas, such as escaped fish, amphibians, or other species, which can consume the feed that left the masculinization hapa and accumulate MT in the adipose tissue over several hormonal treatments (Lone and Ridha, 1993; Mlalila et al., 2015). Even piscivorous birds can feed on larvae or larger fish that have ingested hormone-containing feed outside the hapas, and it is not known whether this can cause physiological changes in these and other predatory animals that eventually visit ponds in search of food (Murray et al., 2016; Murray et al., 2017). Thus, although MT does not pose a risk in direct consumption of tilapia, it is assumed that its bioaccumulation in other organisms may, in the long term, indirectly interfere with human health or in the environment (Baroiller and D'Cotta, 2018).

Therefore, masculinization in ponds presents serious risks to the environment and can represent a potential danger to living beings, including humans. Therefore, carrying out masculinization in close systems, such as the recirculating aquaculture system or the biofloc technology system, is a sustainable possibility that has been tested with the aim of reducing environmental impacts. In BFT, there is even the possibility of eliminating

the release of hormones into the environment due to the rational use of water in the system (Costa e Silva et al., 2022). Furthermore, in BFT it is possible to reduce the fluctuation in water temperature throughout the year due to the use of agricultural greenhouses and heaters, which can maintain water at adequate levels during the masculinization phase of Nile tilapia (Baroiller and D'Cotta, 2018).

To date, there are no completely established protocols for the masculinization of Nile tilapia in bioflocs. There is still no definition, for example, of the ideal stocking density and concentrations of MT in the feed (and its binomial with temperature) to achieve high masculinization rates in this production system. Some research has already provided some preliminary clarification on these and other issues. Costa e Silva et al. (2022) hypothesized that masculinization in BFT could be influenced by the presence of bioflocs, since they are feed available in large quantities for the larvae and could reduce the intake of hormone in the ration. Therefore, they assumed that higher concentrations of methyltestosterone in the diet could be necessary, together with a reduction in feeding frequency, so that the larvae could consume the dose of hormone necessary for masculinization. However, in this study, considerable rates of non-male individuals were found at concentrations greater than 60 mg · Kg^{-1} of feed, while at this concentration, ≥94% of male individuals were obtained. The authors attributed these results to the paradox of sexual inversion, in which the opposite of masculinization occurs, forming non-male individuals (Guerrero, 1975; Cruz and Mair, 1994; Pandian and Sheela, 1995; Beardmore et al., 2001; El -Sayed, 2006). When there is excess MT intake, the activity of the aromatase enzyme can be increased, causing excess androgen to be converted to estrogen (Varadaraj, 1994), resulting in a greater number of non-males.

Therefore, it would be important to verify the ideal concentration of MT in the diet for masculinization in BFT, which would not be reduced to the point of not masculinizing a high proportion of individuals. Furthermore, whether the biofloc system would promote degradation of MT dissolved in water, reducing the possibility of negative environmental impacts. Thus, in this study, it was verified the possibility of using lower concentrations of MT (30, 40, 50 and 60 mg · Kg^{-1} of feed) in BFT, under controlled temperatures around 28 ◦C, aiming to reduction of possible environmental impacts of masculinization of Nile tilapia.

3.3 Material and methods

3.3.1 Experimental design

The experiment was carried out at the Aquaculture Laboratory (LAQUA) of the Veterinary School of the Federal University of Minas Gerais (UFMG). All procedures were previously reviewed and approved by the Counsel of ethical practices in animals of the Federal University of Minas Gerais (CEUA) under protocol number 66/2023.

For the experiment, Nile tilapia larvae (11.15 ± 1.06 mg and 0.93 ± 0.12 cm) were used from spawning and hatching, on the same day, of 6 females, to avoid family × treatment interaction effects. The breeding scheme that gave rise to the spawning's included broodstock of the Chitralada lineage (average weight of 523.33 ± 40.41 g), from the ninth generation of the genetic improvement program for weight gain (more details are available in Cavatti Neto et al., 2023) from the NGT-Aqua (Nutrition, Genetics and Technology for Aquaculture) research group, belonging to the Aquaculture Laboratory/ UFMG, in which 80 females and 64 males were allocated to hapas in the proportion of 10 females and 8 males each. After the reproduction period (7 days), the females that reproduced were separated individually into incubation hapas.

Soon after the yolk sac absorption, the larvae of 6 females that were at the same stage of development were transferred to 24 polyethylene tanks (50 L useful volume), containing 50% of matured biofloc (total ammonia 0.25 ± 0.06; nitrite 0.41 ± 0.07 and nitrate ± 160.56 ± 51.24) and 50% of clear water. A total of 2,880 larvae were distributed in the tanks (120 larvae · $tank^{-1}$) and it was ensured that each tank of each treatment received the same number of larvae from each of the 6 females. The experimental design was in a completely randomized design with 6 treatments and 4 replicates: five treatments with total water renewal after the 28 days of masculinization period with MT in the diet (0, 30, 40, 50 and 60 mg · Kg^{-1}) and a treatment without total water renewal after the 28 days of masculinization period with a diet containing 30 mg of MT · Kg^{-1}.

The tanks were in an agricultural greenhouse and 100-Watt thermostat heaters were also used to maintain the temperature at 28 °C, within the larvae's thermal comfort range. Aeration was maintained by radial blowers connected to microporous hoses installed in each tank to maintain adequate oxygen levels for the species. In addition, perforated bags containing 450g of crushed bivalve mollusk shells were placed in each

tank, as a limestone source, to maintain alkalinity at levels suitable for nitrifying bacteria and the pH close to neutrality.

An initial biometry was carried out with 20 animals, randomly removed from each experimental unit, establishing the initial stocking density of 2 larvae · L^{-1} (n = 100 larvae · $tank^{-1}$). The 20 larvae were weighed together on an analytical balance (©Marte Científica, Brazil), with a precision of 0.001 g. Then, the larvae were euthanized with eugenol (180 mg · L^{-1} for 10 min.) to have their length measured.

After 28 days of feeding with the experimental diets, the fingerling (except for the 30 mg MT · Kg^{-1} of feed treatment without water renewal after 28 days) were transferred to a second battery of tanks, with the same specifications and equipment. of the first battery, containing new biofloc water (previously matured and free from MT) to ensure that there was no influence of any MT residues in the water on the masculinization of the tilapias after 28 days of exposure to treatments, and the fingerlings were kept in the second battery of tanks for approximately 15 days to reach an adequate size for sexing. The water of the tanks of the first battery were maintained with the same experimental conditions during masculinization (aeration, shells, and thermostats) to be analyzed for hormone residues.

The Nile tilapia larvae were fed with a commercial ration (Propescado-Nutriave Foods) containing 55% crude protein, 12% moisture, 10% ether extract and 15% ashes, enriched with MT. Each experimental diet received the masculinizing hormone using 99.5% P.A ethyl alcohol as a vehicle for 17α-methyltestosterone. The solution was previously prepared by weighing the hormone and diluting it in 200 mL of ethyl alcohol. After preparing the solution, the liquid was distributed over the diet using a 500 ml spray bottle. Then, the diet was stored away from light to evaporate the alcohol. The diets were identified and stored in a freezer at -20 °C, protected from light, to preserve MT throughout the experiment.

In the period after masculinization, the fish received a hormone-free diet until they reached an adequate size for sex identification analysis using the aceto-carmine technique (Guerrero and Shelton, 1974).

The larvae were fed five times a day at a feeding rate of 30%, 25%, 20% and 15% of body weight in the first, second, third and fourth week, respectively. Feeding correction

was guided by weekly biometrics, carried out with samples from 20 larvae per tank, estimating a probable weight gain in the following week based on the weight gain of the previous week and a mortality of 3% throughout the entire experiment. However, at the end of the experiment, as body weight gain and survival did not differ between treatments, the amount of feed offered did not differ either (close to 79 g of feed for each experimental unit during the entire period of 28 days).

Considering the treatment of 60 mg of MT · Kg^{-1} of diet, as a reference, the amounts of feed and hormone offered to each larvae were, respectively, 3.35 mg of feed and 201.24 ng of MT · day^{-1} (1,408.68 ng · $week^{-1}$) for each larvae, in the first week; 20.81 mg of feed and 1,248.75 ng of MT · day^{-1} (8,741.25 ng · $week^{-1}$) for each larvae, in the second week; 41.89 mg of feed and 2,513.40 ng of MT · day^{-1} (17,593.80 ng · $week^{-1}$) for each larvae, in the third week; and 53.84 mg of feed and 3,230.46 ng of MT · day^{-1} (22,613.22 ng · $week^{-1}$) for each larvae, in the fourth week.

3.3.2 Water quality

During the entire experimental period, there was no water renewal or solids removal from the tanks, with the volume of water lost through evaporation being supplemented, daily, with clear water from an artesian well.

Temperature, pH, oxygen, and salinity measurements were taken three times a week in the tanks of all treatments. The nitrogenous compounds total ammonia nitrogen (TAN), non-ionized ammonia (NH_3) and nitrite (NO_2^-) were measured twice a week. Alkalinity and settleable solids (SS) analyze were carried out once a week, and nitrate (NO_3^-), total nitrogen (N), total suspended solids (TSS), phosphate and carbon analyze were carried out from initial and final samples of the water from each tank.

Dissolved oxygen levels (mg · L^{-1}) were measured using an AT 155 oximeter (Alfakit®, Florianópolis, Santa Catarina, Brazil); and pH, salinity (g · L^{-1}) and temperature (ºC) were monitored using a multiparameter probe (Hanna®, Barueri, São Paulo, Brazil). The levels of total ammonia nitrogen (TAN) and nitrite (NO_2^-) were measured according to the methodologies established by UNESCO (1983) and Bendschneider and Robinson (1952), respectively, while the nitrate (NO_3) was quantified using the method described by Monteiro et al. (2003).

To determine non-ionized ammonia (NH_3 UIA- N), the formula $NH_3 = [NH_3 + NH_4^+]/[1 + 10^{(pKa-pH)}]$ was used, where pKa = 0.09018 + 2729.92/(273 + T) (Liu, 2008) and was expressed in mg · L^{-1}. To calculate total nitrogen, the amount of nitrogen (N) present in the nitrogenous compounds non-ionized ammonia, ammonium ion, nitrite and nitrate was added, considering their molecular weights 18.04, 17.031, 46.01 and 62.00 mg · L^{-1}, respectively, and the molecular weight of nitrogen 14.01 mg · L^{-1}. Thus, N total = $N\text{-}NH_3 + N\text{-}NH_4^+ + N\text{-}NO_2^- + N\text{-}NO_3^-$.

The determination of phosphate ions was carried out according to the method established by Murphy and Riley (1962).

Alkalinity was measured using the methodologies proposed by APHA (1998). Settable solids (SS) were quantified using Imhoff cones (Avnimelech, 2006). Total suspended solids (TSS) were measured after collecting 20 ml of water, with subsequent filtration through GF50-A glass fiber filters, which were then dried and weighed to quantify the retained material. For carbon analysis, the samples were prefiltered through 0.7 μm fiberglass membrane (Whatman GF/F) and filtered through 0.22 μm cellulose membrane (Millipore®). After the filter process, the samples were stored in acid-washed amber borosilicate bottles refrigerated at 4 ºC in the dark. Total organic carbon (TOC) was determined with a TOC analyzer (Shimadzu TOC-L). The concentration of total organic carbon from the initial mixed water (mature biofloc + clear water) that was used to fill the tanks was 17.32 mg · L^{-1}.

3.3.3 Growth performance, survival, and masculinization rate

The growth performance of tilapia was evaluated by the final body weight (BWf), final body length (BLf), specific growth rate (SGR), Fulton condition factor (CF) and survival (S), at the 29th day, in the morning, before the first feeding of the day, as follows:

final body weight (BWf) = mean of the mass (g) of 20 larvae randomly picked per experimental unit;

final body length (BLf) = mean of the length (cm) of 20 larvae randomly picked per experimental unit;

specific growth rate (SGR) = 100 × (log (final weight) – log (initial weight)) / days of experiment;

condition factor (FC) = mass (g) × total length^{-3} (cm) x 100;

survival (S) = (final number of individuals / initial number of individuals) x 100.

After a period of 28 days of experiment, the fingerlings from each treatment were removed, as they reached the minimum size of 3.5 cm for sexing, to verify the effect of MT concentrations on masculinization. For this analysis, tilapias were euthanized via eugenol-induced overdose (180 mg · L^{-1} for 10 min.) (Vidal et al., 2008). The fingerlings were fixed in Bouin's liquid for 24 hours and dehydrated in 70% ethyl alcohol. Subsequently, their gonads were removed and analyzed under an optical microscope, using 40x objectives, using the aceto-carmine technique (Guerrero and Shelton, 1974).

3.3.4 MT residues in the water

The evaluation of MT input into the system was carried out based on the amount of feed offered and the concentration of MT used in each treatment. Subsequently, this value was adjusted to a tank of 100 m³, multiplying the result by 2000. Thus, we have:

MT input (mg) for 100 m³ = feed offered (Kg) × [ ] of MT × Kg^{-1} of feed × 2,000

Water samples from each tank were collected 12 hours after the last feeding with hormone-containing food and for the subsequent 9 days. MT analysis in water was first validated in-house so that water samples from the tanks could be evaluated, following the procedures below:

Chemicals and reagents

Acetonitrile and methanol were HPLC grade and purchased from Sigma Aldrich (St. Louis, MO, USA). Water was purified in-house with a Milli-Q water purification system (18.2 Ω, Millipore Co., MA, USA). All other chemicals were higher than analytical grade and obtained from commercial sources. Methyltestosterone (MT) standard were purchased from Active Pharmaceutica Ltda (Palhoça, SC, Brazil) with purity ≥ 99%. The standard stock solution was prepared in methanol at a concentration of 1000 μg · mL^{-1}. The working solutions were prepared through appropriate dilutions of the stock solutions. All the solutions were stored at -20 °C for subsequent use.

Sample preparation

Blank samples of biofloc water were fortified with MT for the validation procedures of the analytical method. For the extraction equal volume of methanol and biofloc water were added to test tubes and vortex mixed for 1 minute. Samples were than centrifuged at 4500 rpm for 5 minutes and filtered through syringe filter CHROMAFIL® PTFE, 15 mm, 0.2 µm (Macherey-Nagel GmbH & Co KG, Düren, Nordrhein-Westfalen, Germany). The extracts were used for chromatographic analyses.

Preparation of calibration standards

Appropriate amounts of stock/working solution were spiked into blank matrix extracts to prepare the series of matrix matched calibration standards of 0.1, 0.2 and 0.4 $\mu g \cdot mL^{-1}$.

Instrument and HPLC conditions

High performance liquid chromatography (HPLC) analyses were performed using an JASCO LC-2000Plus HPLC System (Jasco International CO., LTD., Hachioji, Tokyo, Japan) equipped with a quaternary gradient pump (PU-2089S), 4-line degasser, autosampler (AS 2059), column oven (CO-2065), UV/VIS detector (UV-2075) and fluorescence detector (FP-2020). Chromatographic separations were performed in reverse phase using a C18 column (150 x 4.6mm, 5µm particle size) (Supelcosil™ LC-ABZ, Supelco, Bellefonte, PA, USA). The mobile phase used was acetonitrile:methanol:water (50:10:40 v/v/v) in isocratic mode. The injected volume was 50 µL, the flow was 1.0 mL per minute, and the column temperature was set to 30 °C. MT was detected at 245nm using a UV detector. Peak area values were plotted against concentration and calibration curves for MT were constructed using least squares method for linear regression. ChromNAV 2.0 software was used for data acquisition and data processing. Each sample was analyzed in triplicate.

Method validation parameters

Selectivity is the ability that an analytical method could differentiate the target analytes from other unrelated components present in the sample. This analysis was carried out via evaluating six batches of blank biofloc water. The chromatograms of blank samples were compared with those that spiked with MT to investigate whether or not the target analyte had the same retention time with endogenous interference compounds from samples.

Matrix effect is the effect on an analytical method caused by all other components of the sample except the specific compound to be quantified. A calibration curve was constructed by linear regression with the calibration standards in solvent solution (methanol). Next, another calibration curve was constructed from the measurement data of the matrix matched calibration standards.

Linearity of both curves were studied with *F*- and *t*-test then the evaluation of matrix effect was done by assessing the parallelism of the response curves. The significance level (α) was 0.05.

The calibration curve of MT was constructed by analyzing the biofloc water extract samples spiked with the analyte at gradient concentrations on three days. The peak-area values were plotted against the concentration and calibration plots were constructed using a weighted least-squares linear regression. The correlation coefficient (r) and coefficient of determination (R^2) were also calculated.

The limit of detection (LOD), the lowest concentration that produces a response differentiable from the noise, were determined using the signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) were calculated as the minimum concentration calculated using the signal-to-noise ratio of 10:1. This analysis was performed by evaluating three replicate samples.

The precision of the method was assessed by means of repeatability studies and intra-laboratory reproducibility. The intra- and inter-day values were evaluated at three concentration levels in six replicates within a day and three successive days. The precision was expressed as the relative standard deviation (RSD) to detect the distribution of measured concentration.

The accuracy of the method was assessed by the spike recovery method. Three concentration levels in six replicates of MT were added to matrix (spiked) and then the

analysis was performed on the spiked material, from the sample preparation through chromatographic determination. The comparison of the amount found versus the amount added provided the recovery (%) of the method.

3.3.5 Statistical evaluation

The data were submitted to analysis of variance (ANOVA), followed by Tukey (CV < 10%), Student-Newman-Keuls (CV of 10 to 15%) or Duncan (CV > 15%) tests, when the assumptions of normality and homogeneity of variances were met, according to the Shapiro-Wilk and Levene tests, respectively. Differences were considered significant when $p < .05$. For growth performance, linear regression models were also adjusted. When the assumptions of normality and homogeneity were not met, the data were submitted to the Kruskal-Wallis non-parametric test ($p < .05$). The chi-square test was used to compare masculinization proportions. Statistical analyzes were performed using the InfoStat program (Di Rienzo et al., 2015) and R software (R Core Team, 2021).

3.4 Results

3.4.1 Water quality

Throughout the experimental period, there were no differences between the mean values of the water quality variables temperature (28.52 ± 0.65 °C), pH (6.15 ± 0.12), oxygen (7.18 ± 0 .21 mg · L^{-1}), total suspended solids (0.04 ± 0.01 mg · L^{-1}), settleable solids (5.81 ± 2.20 mg · L^{-1}), alkalinity (36.64 ± 5.01 mg $CaCO_3$ · L^{-1}), total ammonia nitrogen (0.17 ± 0.08 mg · L^{-1}), toxic ammonia (NH_3) (0.098 ± 0.059 µg · L^{-1}), nitrite (0.84 ± 0.53 mg · L^{-1}), nitrate (245.97 ± 85.00 mg · L^{-1}), total nitrogen (57.52 ± 19.49 mg · L^{-1}), salinity (0.36 ± 0.02 g · L^{-1}), phosphate (0.04 ± 0.01 mg · L^{-1}) and final total organic carbon (68.74 ± 10,11 mg · L^{-1}) in the masculinization of tilapia at different MT concentrations in the feed ($p > .05$) (Table 1).

Table 1. Water quality variables (mean or median) and their coefficients of variation (CV) or minimum and maximum values (in parentheses) for Nile tilapia reared in biofloc technology (BFT) during 28 days of masculinization under different concentrations of 17α-methyltestosterone (MT) in the diet (0, 30, 40, 50 and 60 mg · Kg^{-1} of feed) with water change on the 28th day, at the end of hormonal treatment, and maintenance of the same water after the 28th day for treatment with 30 mg · Kg^{-1} of feed (C30) until masculinization analysis.

Variable	0	30	40	50	60	C30	CV (%)
Temperature (°C)*	29.10[a]	28.53[a]	28.22[a]	28.64[a]	28.66[a]	28.00[a]	2.18
pH*	6.03[a]	6.10[a]	6.16[a]	6.16[a]	6.18[a]	6.28[a]	1.72
Oxygen ($mg \cdot L^{-1}$)****.	7.56[a] (7.5-7.64)	7.90[a] (6.81-7.14)	7.11[a] (6.88-7.28)	7.20[a] (7.09-7.26)	7.16[a] (7.15-7.21)	7.04[a] (6.98-7.17)	-
Settleable solids ($mL \cdot L^{-1}$)**	5.81[a]	7.04[a]	6.00 [a]	5.67[a]	6.00[a]	4.33[a]	39.7
Total suspended solids ($mg \cdot L^{-1}$)**	31.25[a]	33.50[a]	28.25[a]	40.25[a]	34.00[a]	47.00[a]	38.13
Alkalinity (mg de $CaCO_3 \cdot L^{-1}$)***	34.84[a]	39.06[a]	38.44[a]	34.38[a]	35.31[a]	37.81[a]	14.32
Total ammonia ($mg \cdot L^{-1}$)****	0.16[a] (0.12-0.17)	0.13[a] (0.10-0.14)	0.12[a] (0.10-0.33)	0.21[a] (0.13-0.26)	0.12[a] (0.05-0.23)	0.21[a] (0.2-0.41)	-
Non-ionized ammonia (NH_3) ($\mu g \cdot L^{-1}$)****	0.053[a] (0.04–0.077)	0.081[a] (0.052-0.13)	0.081[a] (0.64-0.087)	0.100[a] (0.065-0.32)	0.074[a] (0.063-0.11)	0.13[a] (0.094-0.21)	-
Nitrite (NO_2) ($mg \cdot L^{-1}$)****	0.78[a] (0.47-1.31)	0.79[a] (0.30-1.65)	0.98[a] (0.66-1.62)	1.18[a] (0.38-2.60)	0.66[a] (0.37-1.12)	0.64[a] (0.44-1.0)	-
Nitrate (NO_3) ($mg \cdot L^{-1}$)**	207.76[a]	183.81[a]	261.04[a]	262.44[a]	278.86[a]	281.93[a]	35
Total nitrogen (N) ($mg \cdot L^{-1}$)**	48.58[a]	43.23[a]	61.50[a]	62.00[a]	64.48[a]	65.34[a]	34.32
Salinity ($g \cdot L^{-1}$)****	0.38[a] (0.36-0.39)	0.36[a] (0.34-3.51)	0.36[a] (0.34-0.38)	0.36[a] (0.35-0.38)	0.36[a] (0.33-0.37)	0.36[a] (0.34-3.50)	-
Phosphate (PO_4^{3-}) ($mg \cdot L^{-1}$)****	0.04[a] (0.03-0.06)	0.04[a] (0.03-0.04)	0.05[a] (0.03-0.06)	0.05[a] (0.04-0.06)	0.05[a] (0.03-0.06)	0.04[a] (0.04-0.06)	-
Initial total organic carbon ($mg \cdot L^{-1}$)***	17.32	17.32	17.32	17.32	17.32	17.32	-
Final total organic carbon ($mg \cdot L^{-1}$)***	64.88[a]	68.81[a]	69.80[a]	76.74[a]	63.0[a]	69.22[a]	14.71
Total organic carbon difference (final – initial) ($mg \cdot L^{-1}$)***	47.56[a]	51.49[a]	52.48[a]	59.42[a]	45.68[a]	51.90[a]	19.67

*Means in the same line did not differ according to ANOVA and Tukey test ($p > .05$). **Means in the same line did not differ according to ANOVA and Duncan's test ($p > .05$). *** Means in the same line did not differ according to ANOVA and SNK test ($p > .05$). ****The median in the same line did not differ according to the Kruskal-Wallis test ($p > .05$). Reference values: a El-Sayed (2019). b Wedemeyer (1996). c Hargreaves (2013). d Avnimelech (2009). e Monsees et al. (2017). f Alvarenga et al. (2018).

3.4.2 Growth performance, survival, and masculinization rate

As expected, the growth performance variables and survival were not changed ($p > .05$) due to differences in the MT concentrations used. At the end of the masculinization period (28 days), the average survival of the treatments was 88 ± 9.68%, final body weight of 418.87 ± 93.48 mg, final length of 29 ± 0.33 mm, specific growth rate of 12.88 ± 0.87 % · day^{-1} and condition factor of 1.95 ± 0.13 (Table 2).

There was a significant difference, in the proportion of males, between the control and the other MT concentrations ($p < .05$). However, there were no differences between treatments with concentrations between 30 and 60 mg of MT · Kg^{-1} of feed ($p > .05$) (Fig. 1), including the treatment that did not make a water change at the end. The proportion of non-males (females and intersex) in the control treatment, without the presence of hormones in the feed, was 26.40%, while in treatments containing hormones, the highest value observed was 1.15%. This difference indicates the significant masculinizing effect of the MT hormone.

Table 2. Means of growth performance variables and survival and their coefficients of variation (CV) for Nile tilapia produced in biofloc technology (BFT) during 28 days of masculinization under different concentrations of 17α-methyltestosterone (MT) in the diet (0, 30, 40, 50 and 60 mg • Kg-1 of feed) with water change on the 28th day, at the end of hormonal treatment, and maintenance of the same water after the 28th day to the treatment of 30 mg • Kg—1 of feed) (C30) until the masculinization analysis.

Variable	0	30	40	50	60	C30	CV (%)
Initial body weight (mg)**	11.11	10.07	11.83	11.61	11.34	11.12	8.83
Final body weight (mg)*	372.24	417.62	362.82	447.96	459.66	441.27	23.24
Initial body length (cm)**	0.92	0.92	0.88	0.89	0.91	0.91	3.56
Final body length (cm)**	2.66	2.80	2.64	2.85	2.86	2.78	7.67
Specific growth rate (%•day—1)**	12.59	13.16	12.19	13.03	13.12	13.11	6.97
Condition factor**	1.96	1.87	1.96	1.94	1.95	2.04	6.86
Survival (%) ***	89.67	93.00	84.00	84.50	90.75	89.00	11.66

*Means in the same line did not differ according to ANOVA and Duncan's test ($p > .05$). **Means in the same line did not differ according to ANOVA and Tukey test ($p > .05$). ***Means in the same line did not differ according to ANOVA and SNK test ($p > .05$).

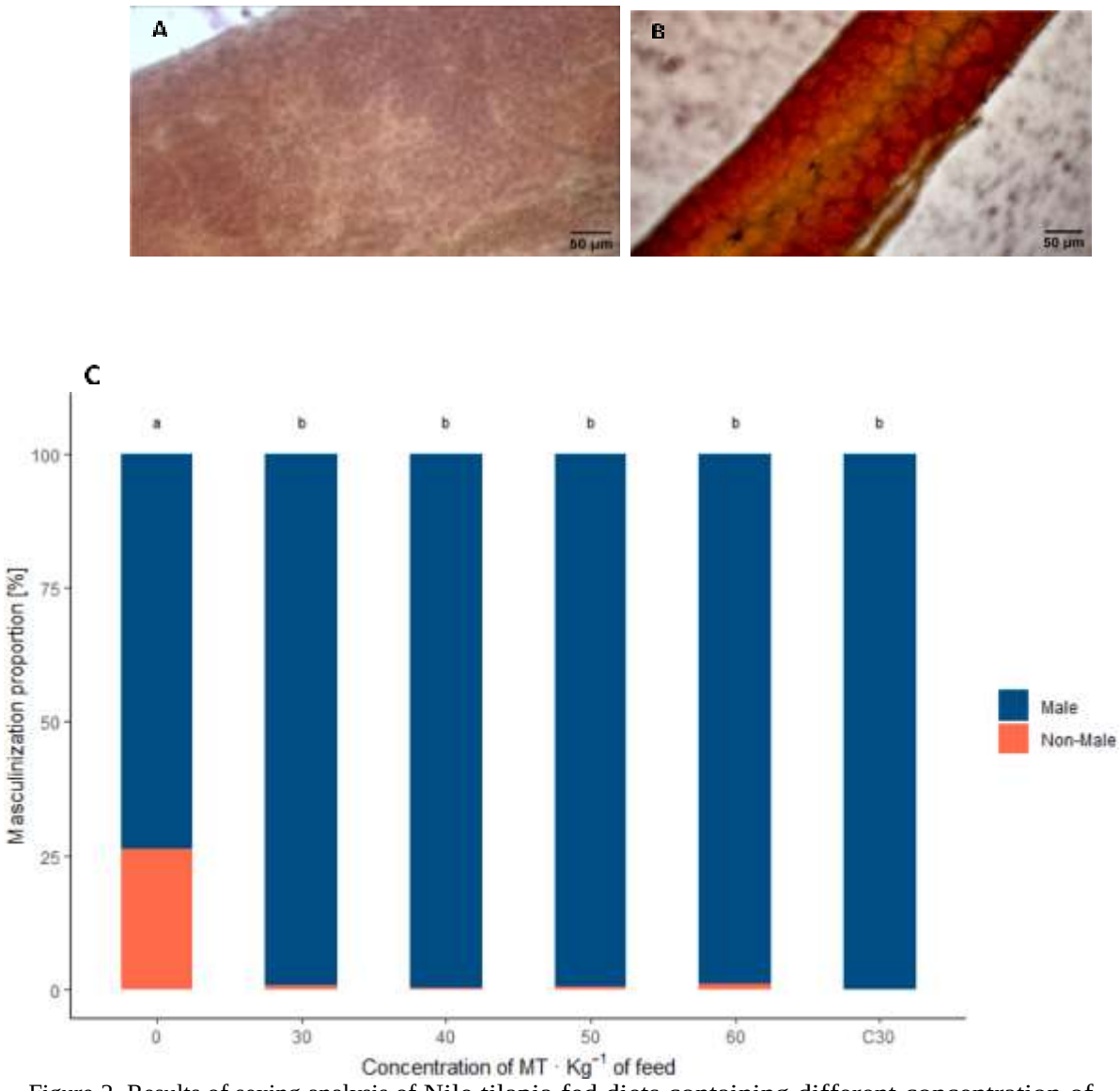

Figure 2. Results of sexing analysis of Nile tilapia fed diets containing different concentration of 17α-methyltestosterone during the masculinization phase. A) Microscopic image of a male gonad subjected to the aceto-carmine technique from an animal in the control group of Nile tilapia produced using biofloc technology (BFT). B) Microscopic image of female gonad subjected to the aceto-carmine technique from a Nile tilapia control animal produced using biofloc technology (BFT). C) Pro- portion of males and non-males (females and intersex) of Nile tilapia during 28 days of masculinization under different concentration of 17α-methyltestosterone in the diet (0, 30, 40, 50 and 60 mg • Kg^{-1} of feed) in biofloc technology (BFT) with water change (without possible hormonal residues in the water) on the 28th day, and with 30 mg • Kg^{-1} of feed maintaining the original water (with possible hormonal residues in the water) after the 28th day (C30) until masculinization analysis. The proportion of non-males obtained for 0, 30, 40, 50, 60 mg and C30 mg of MT • Kg^{-1} of feed was 26. 40, 0.79, 0.42, 0.51, 1.15 and 0.00%, respectively.

3.4.3 MT residues in the water

The amount of MT that would enter the system throughout the entire experiment, when adjusted for a tank of 100m³, would be 4,660.18; 6,213.58; 7,766.97 and 9,320.36 mg of MT for concentrations 30, 40, 50 and 60 mg · Kg^{-1} of feed, respectively.

The results of validation and the analysis of the water samples are presented below.

Method validation

Selectivity

The chromatograms obtained from the blank sample extracts were compared with those obtained from the fortified samples. The signals absence, considering the respective retention time and the signal-to-noise ratio, which could be derived from matrix interfering compounds at the same retention time of the searched analyte (MT), confirmed the good selectivity of the method (Ribani, 2004).

Precision and accuracy

The data to evaluate the precision and accuracy of inter- and intra-day variability for MT levels carried out on samples at three concentration levels were listed in Table 3. The precision in RSD form was in the range of 1.281-7.175. These values were accorded to INMETRO (2020) and located in the acceptable scope within 20%. The accuracy in recovery form was in the range of 89.12-97.49%. These values were in the acceptable scope range of 70-120% (Ribani, 2004; INMETRO, 2020). These promising results demonstrate that this developed method can be successfully applied to the analysis of MT in biofloc water with excellent precision and accuracy.

Table 3. Intra-day and inter-day accuracy (mean ± standard deviation) and precision of methyltestosterone in biofloc water at three different levels.

Methyltestosterone	Intra-day (n = 6)		Inter-day (n = 6)	
Spiked concentration ($\mu g \cdot mL^{-1}$)	Measured concentration ($\mu g \cdot mL^{-1}$)	Precision (RSD, %)	Measured concentration ($\mu g \cdot mL^{-1}$)	Precision (RSD, %)
0.1	0.092 ± 0.007	7.175	0.089 ± 0.006	6.68
0.2	0.196 ± 0.006	2.990	0.195 ± 0.006	3.30
0.4	0.379 ± 0.005	1.281	0.376 ± 0.011	3.01

Calibration curves, LOQ and LOD

The analytical curve of MT showed favorable linearity. The linear regression curve was showed by the equation y=73.951x-58.021, obtaining the correlation coefficient (r=0.999) and determination coefficient (R^2=0.999). Analytical curve was constructed with six different concentration levels of MT (0.025, 0.05, 0.1, 0.2, 0.4, 0.6 $\mu g \cdot mL^{-1}$), which meet the requirements established by the International Conference on Harmonization (ICH), Agência Nacional de Vigilância Sanitária - National Health Surveillance Agency - (ANVISA), and the Group of Pesticide Residue Analysts (GPRA), which recommend a calibration curve with at least five concentration levels (ICH, 1994; ANVISA, 2003). This result is consistent with the Brazilian law which establishes a values variation for R^2 between 0.99 and 0.90 (ANVISA, 2003). The LOQ of MT in biofloc water was calculated as 0.20 $\mu g \cdot mL^{-1}$ in accordance with the S/N = 10. The LOD was measured at the concentration of 0.02 $\mu g \cdot mL^{-1}$ in accordance with the S/N = 3. Consequently, this established method was appropriate to quantify MT in biofloc water.

Matrix effect

In the matrix effect analysis, the analytical curves of the solvent patterns and the patterns in the matrix were compared, finding no significant statistical difference between them. In this way, it was chosen to validate the method with the construction of curves in the solvent.

Determination of MT in biofloc water samples

None of the biofloc water samples were positive for residues of MT.

3.5 Discussion

Hormonal treatment with MT in feed is widely used in the masculinization of Nile tilapia with the aim of preventing premature reproduction, obtaining higher growth rates, uniformity in harvesting and avoiding overpopulation, which causes complications for fish farmers (Singh, 2013; Mlalila et al., 2015; Baroiller and D'Cotta, 2018). However, the way in which masculinization is commonly carried out in tilapia hatcheries, especially in ponds, can generate effluents contaminated with hormones. Masculinization in the biofloc system emerges as a sustainable alternative in this management, reducing environmental impacts, as it allows greater control of effluents. However, there is still no completely defined protocol for masculinization in BFT determining the appropriate concentration of MT in the feed. Based on results from Costa e Silva et al. (2022), who obtained ≥94% of males using 60 mg of MT · Kg^{-1} of feed, the present study demonstrated that it is possible to achieve higher rates of males (>99%) with lower concentrations of MT (30 mg · Kg^{-1} of feed), without changes in the variables of water quality or growth performance, thus reducing impacts on the environment.

3.5.1 Water quality

Water quality mean variables remained at values suitable for biofloc production, not affected by MT concentrations. The combination of slight pH acidity (between 6.03 and 6.28), alkalinity below 60 mg $CaCO_3$ · L^{-1} (between 34.38 and 39.06 mg $CaCO_3$ · L^{-1}), low levels of total ammonia nitrogen (between 0.12 and 0.21 mg · L^{-1}) and nitrite (between 0.64 and 1.18 mg · L^{-1}) and higher levels of nitrate (between 183.81 and 281.93 mg · L^{-1}) in water are indicative of the adequate functioning of the nitrifying activity. During the nitrification process, bacteria release hydrogen molecules, acidifying the medium, and consume carbonates as they transform ammonia into nitrite and, subsequently, nitrite into nitrate (Ebeling et al., 2006; Liu et al., 2018), resulting in low alkalinity values (Table 1). It was not necessary to use organic carbon sources to stimulate the formation of heterotrophic bacteria and bioflocs, as their quantity was sufficient to

maintain the environment with low concentrations of ammonia in the water. Alkalinity was not corrected due to the presence of seashells in the experimental units to slowly release, with the movement of aeration, calcium carbonate from its limestone composition.

The amount of organic carbon added to each experimental tank can be estimated, considering that there was no water change in the entire masculinization period, using the amount of feed offered (79,000 mg of feed for each experimental unit), the proportion of dry matter in the starter feed (88%), the average proportion of organic carbon in the dry matter of starter feed (41.36%; Chatvijitkul et al., 2017) and the average proportion of organic carbon from the starter feed that is not retained in the fish (81.7%; Chen et al., 2015):

$$79{,}000\ mg \cdot 88\% \cdot 41.36\% \cdot 81.7\% \cdot 28\ days^{-1} \cdot 50L^{-1} = 16.78\ mg \cdot L^{-1}$$

When considering that only 16.78 mg of organic carbon was added to the tanks because of feeding, the difference of more than 50 $mg \cdot L^{-1}$ between the final and initial measurements of total organic carbon (Table 2) can be largely attributed to the carbon retention carried out by the algae. This result indicates that BFT system, even over short periods of time, can contribute to the retention of carbon, as has already been suggested by Lee et al. (2023), and presented by other authors for the pond system (Chen et al., 2015; Zhang et al., 2020).

3.5.2 Growth performance, survival, and masculinization rate

The growth performance and survival variables were not affected by the hormone concentrations used. Testing higher concentrations (60, 90, 120 and 150 mg of $MT \cdot Kg^{-1}$ of feed) and two types of feeding frequency (five and eight times) in the masculinization of tilapia, Costa e Silva et al. (2022) observed a reduction in growth the higher the MT concentrations, while survival was not affected. This reduction in growth may be related to the catabolic effect that MT can cause when ingested in high concentrations (Donaldson et al., 1979; Lone and Matty, 1983).

David-Ruales et al. (2019) obtained a tilapia masculinization rate in BFT of 61%, using the concentration usually practiced in ponds of 60 mg MT per Kg of feed, a fixed feeding rate of 10% of body weight throughout the entire experiment at an average

temperature of 26.62 °C. Costa e Silva et al. (2022), however, using the same concentration and the same feeding protocol as in the present study, they obtained a rate of 94% of males, at a temperature of 26.54 °C. In our study, masculinization rates were not affected by different concentrations and remained high (≥ 99%). In the study of David-Ruales et al. (2019) the authors considered that the excess of settleable solids would be responsible for the low masculinization rate, however, the authors did not consider that this result may be related to the insufficiency of daily MT offer, due to the low feeding rate used throughout the period of masculinization. Costa e Silva et al. (2022), testing concentrations above 60 mg of MT per Kg of feed, found that there was an association between increased concentration and a reduction in male individuals, and associated this result with the paradoxical effect of sexual inversion. It is known that excessive MT intake can cause the paradoxical effect of sexual inversion, in which there is a greater formation of non-male individuals (Guerrero, 1975; Cruz and Mair, 1994; Pandian and Sheela, 1995; Beardmore et al., 2001; El-Sayed, 2006).

In our experiment, due to higher average temperatures (28.53 °C) than those described in the studies already mentioned, we had a proportion of around 76.4% of males in the control, a value above the expected proportion (50%) at a lower temperature (Fig. 1), which could mean that in treatments with concentrations below 60 mg of MT · Kg^{-1} of feed, the temperature may also have been responsible for increasing the proportion of males (Baroiller and D 'Cotta, 2018), achieving better results than those of Costa e Silva et al. (2022). Furthermore, it is possible that there was a synergistic effect between these two factors (MT and temperature), and it could even be that, due to the acceleration of metabolism at higher temperatures, there was a greater consumption of feed, and, therefore, a greater intake of MT.

Due to the high masculinization values obtained at the lowest concentration (30 mg of MT · Kg^{-1} of feed) in the present study, we have an indication that it is possible to perform masculinization in BFT with concentrations lower than 60 mg of MT · Kg^{-1} of feed and not larger as suggested by Costa e Silva et al. (2022). Perhaps, values even lower than 30 mg · Kg^{-1} of feed may be more effective depending on the water temperature. Therefore, studies that evaluate different concentrations of MT in feed, in interaction with

different temperatures in the BFT water, are important for a clearer definition of adequate concentrations of the hormone.

The masculinization rate (100%) found in treatment C30, which used a concentration of 30 mg of MT · Kg^{-1} of feed and kept the fish in the same water after treatment with hormone in the feed, did not differ from the rate found in the treatment who used a concentration of 30 mg · Kg^{-1} of feed (99.21%) and placed the fish in new water after the experimental period. The stay of the fingerlings in the same water where the feed was offered (C30) could have increased the proportion of males in relation to the treatment with the same concentration and replacement of the water with a new one, due to the possible residue of MT in the water; or increase the proportion of non-males due to the sexual inversion paradox if there was an excessive accumulation of MT residues. However, since any MT residue was detected 12 hours after the last supply of feed with hormone, these situations are unlikely.

3.5.3 MT water residues

Using a concentration of 30 mg of MT · Kg^{-1} of feed in the masculinization of Nile tilapia in BFT, and extrapolating to a volume of 100 m^3, it was possible to verify a reduction of 4,660.18 mg of MT when comparing the same procedure being carried out using a concentration of 60 mg of MT · Kg^{-1} of feed.

The analysis of MT in the water, 12 hours after the last supply of feed with hormone, did not detect the presence of MT. This result indicates that maintaining the same water in the post-treatment period will not affect the masculinization rate of Nile tilapia, in the range of concentrations studied, since a natural degradation of MT probably occurs in a period of less than 12 hours.

The lack of MT in the BFT system after the masculinization period probably occurs due to the influence of various processes present in the environment, including the adsorption of MT to sedimented solid particles (Ong et al., 2012), photodegradative action (Shore and Shemesh, 2003; Biswas et al., 2013) or the biotransformation of MT through microbiological activity (Kolok and Sellin 2008). Some microorganisms present in aquaculture systems can assimilate steroids and use them as a source of carbon and energy (Green and Teichert-Coddington, 2000). Among the microorganisms found in

aquaculture production, Pimelobacter simplex bacteria, belonging to the genus *Nocardioidaceae*, and bacteria from the genus *Mycobacterium* can convert MT into methandrostenolone (ME2) (Homklin et al., 2012; Baroiller and D'Cotta, 2018.). Therefore, this study paves the way toward investigating the presence of these microorganisms in BFT and the use of aerobic conditions of the system to efficient remediation of MT-contaminated sediments.

The absence of MT in the water 12 hours after the end of the Nile tilapia hormonal treatment period in the BFT system is an important advance from an environmental and marketing point of view. These results reinforce the sustainable nature of this system, which can contribute to reducing future trade barriers that some markets impose due to the possible impact that masculinization carried out in ponds can cause. Furthermore, a consumer public that is increasingly informed and concerned about environmental issues may prefer to consume foods that do not cause environmental impacts.

3.6 Conclusion

Results show that the intra-laboratory validation of the analytical method for detection and quantification of methyltestosterone by high performance liquid chromatography using 245 nm UV absorbance have had an excellent applicability for the residues analysis in biofloc water.

This study demonstrated that it is possible to perform the Nile tilapia masculinization procedure in the BFT system, reducing the most used MT concentration (60 mg · Kg^{-1}) by half (30 mg · Kg^{-1}), at a mean temperature of 28.52 ◦C, without MT residues in the water 12 hours after the end of hormonal treatment.

3.7 Acknowledgements

This research received financial support from FAPEMIG, CAPES and CNPq (National Council for Scientific and Technological Development).

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4. CAPÍTULO III- CONTRIBUTION OF WATER TEMPERATURE IN A ZERO WATER EXCHANGE BIOFLOC SYSTEM ON THE REDUCTION OF METHYLTESTOSTERONE USED DURING MASCULINIZATION OF NILE TILAPIA (O*reochromis niloticus*)

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4.1 Abstract

Production in closed systems, such as biofloc technology (BFT), allows controlling the water temperature, uses a smaller volume of stored water and generates a smaller volume of effluents, being an alternative system for the in pond masculinization process for Nile tilapias, which demands water changes in large volumes, generating a large amount of effluents that may contain hormone residues and causes environmental impacts. Recent studies have demonstrated the possibility of reducing methyltestosterone (MT) concentrations by half in tilapia masculinization using BFT with zero water exchange (30 mg against 60 mg of MT · Kg^{-1} of feed, the concentration generally used in pond masculinization). However, to date, the contribution of temperatures below those suggested in the literature to high masculinization rates in Nile tilapia has not yet been verified in BFT. Since temperature plays a crucial role in consumption, chemical reactions and metabolism, it was hypothesized that its elevation could favor masculinization in BFT with zero water exchange, even if temperatures recognized as masculinizing (>32 °C) are not reached, and thus contribute to lower MT concentrations achieving higher masculinization rates, further maximizing the already known sustainable role of BFT with zero exchange in tilapia masculinization. This study evaluated the masculinization rates of Nile tilapia larvae (post yolk sac absorption) in a zero water exchange BFT system, in two controlled temperatures of around 25 and 28 °C combined with concentrations of MT in the feed (0, 10, 20, 30 and 40 mg · Kg^{-1} of feed) lower than those commonly recommended for masculinization in ponds and also BFT (60 mg · Kg^{-1} of feed) with the possibility of reducing this impact on the environment. Nile tilapia larvae were fed five times a day for a period of 28 days. The experimental design was entirely randomized using 3 replicates for each treatment (3 replicates x 2 temperatures x 5 MT concentrations = 30 tanks of 50 liters). The stocking density applied was 2 larvae · L^{-1} (100 larvae · $tank^{-1}$). After the hormonal treatment period, the fry remained until they reached a viable size for sexing. The water quality and growth performance variables did not diverge between MT concentration levels in the diet ($p > .05$). However, the larvae grew 2.7 times higher in 28 °C than 25 °C. The treatments without hormone in the diet (control with 0 mg · Kg^{-1} of feed) presented 68.6% and 64.9% of males at temperatures 25 °C and 28 °C, respectively, not diverging from each other, and the treatments that received hormone in the feed showed higher masculinization rates of over 98.7% and

96.9%, at temperatures 25 °C and 28 °C, respectively. At 25 °C the masculinization rates did not differ from each other ($p > 0.05$) and from 28 °C at the same MT concentrations. Therefore, it is feasible to use an even lower concentration (10 mg of MT · Kg^{-1} of feed) in a zero-water exchange BFT, regardless of these temperatures. However, the lower temperature made the masculinization process even more sustainable, as the fish required smaller amounts of feed due to growth deceleration, and therefore there was less MT input into the system. Furthermore, after 2 hours of the last hormone feeding, the presence of MT was not detected in the water, which increases the prospect of adoption of this system by tilapia producers for the practice of masculinization. These results provide support for a paradigm shift in the sustainability of tilapia production and are even closer to what could become a protocol aimed at masculinization in BFT with zero water exchange.

Key-words: bioflocs, tilapia hatchery, metiltestosterone, temperature, masculinization, environmental sustainability.

4.2 Introduction

For fish producers, the sexual precocity of tilapia is not interesting, as it generates heterogeneous batches, loss of population control, incorrect feeding, reduced growth (Farahmand et al., 2007; Baroiller et al., 2014), as well as excess management and damage to water quality. The production of monosex tilapia is a form of control of these negative impacts of reproduction. There are various techniques for reaching populations of only one sex, and one of these uses male and female steroid sex hormones offered in the diet (Karsli, 2021). In the case of tilapia, the choice is to produce male individuals, obtained using male hormones, a technique most applied due to its easy application, low cost, and greater effectiveness (Baroiller and D'Cotta, 2018; Costa e Silva et al., 2022). The hormone most used in this technique is 17α-methyltestosterone (MT), due to its high inversion capability in male individuals (Sarker et al., 2022).

Several studies have proven that the use of MT in tilapia production does not contaminate the meat of the fish produced (Rothbard et al., 1990; Curtis et al., 1991; Piferrer, 2001) and does not pose a risk to human health (Baroiller and D 'Cotta, 2018; Thanasupsin et al., 2021). However, this procedure has environmental impacts as it is

generally carried out in hatcheries, where the exchange of water and disposal of effluents can contaminate natural bodies of water (Baroiller and D'Cotta, 2018; Ramírez et al., 2024). MT degradation and biotransformation pathways, which occur in the environment and in fish, have not yet been completely elucidated (Lone and Ridha, 1993; Mlalila et al., 2015). Therefore, it is not yet known whether the metabolites formed after the metabolization of MT continue to have any potential for steroid action.

In a recent study, Ramírez et al. (2024), using a water temperature of 28 °C, managed to reduce the use of MT in the masculinization of tilapia in a zero water exchange BFT system to 30 mg · Kg^{-1} of feed, half of what is commonly used in ponds, reaching 99% of males and finding an absence of MT residue in the water after 12 hours after the last hormone-containing feeding. As a result, the environmental problem related to the use of MT in tilapia masculinization was minimized using a BFT system with zero exchange, thus opening new paths towards a sustainable tilapia hatchery.

Temperature directly affects the environment and the metabolic rates of aquatic organisms (Rubalcaba, 2020), that is, it interferes both with water parameters and with the well-being, consumption, and performance of fish. Among the abiotic factors that can interfere with gonadal differentiation, temperature is the one that stands out the most (Baroiller and D' Cotta, 2001; Yao et al., 2021). The explanation for sexual inversion due to temperature in fish lies in the ability of the enzyme cytochrome P450-aromatase to catalyze the conversion of testosterone into 17-β-estradiol, a hormone that acts in the differentiation and development of the ovaries. Temperature treatment has a negative regulatory action on the expression of the gene responsible for expressing this enzyme, that is, causing it to initiate a process of development of male sexual structures associated with the development of functional testes. In tilapia larvae subjected to a temperature of 35 °C during the differentiation period, masculinization of XX females occurs due to the blockage of this enzyme (Guiguen et al., 1999; Baroiller and D'Cotta, 2001).

Recently, the use of heat treatment for masculinization has been gaining prominence, however, the temperatures used in this technique are generally well above the range between 27° and 30 °C, considered ideal for Nivelle et al (2019), resulting in high mortalities (Borges et al., 2005; Dias-Koberstein et al., 2006), and raising questions related to animal welfare. Outside the temperature range considered optimal, fish can

experience a stressful situation, leading to physiological dysfunctions that can impair performance and even lead to death (Pörtner et al., 2017; Yang et al., 2021).

At temperatures above the ideal limit, a reduction in fish consumption and growth performance may occur (Islam et al. 2020, Khieokhajonkhet et al. 2022). A study carried out by Zhou et al. (2022), exposed tilapia to a temperature of 36 °C, and observed that there was an increase in ventilatory frequency, a decrease in tolerance to hypoxia and a 21% increase in mortality when compared to tilapia kept in water at 28 °C. Borges et al (2005), comparing the masculinization rates of Nile tilapia of the Chitralada lineage at temperatures 27 °C and 35 °C, achieved rates of approximately 62% and 72%, respectively. Nevertheless, they attributed it to the higher temperature and greater mortality caused by cannibalism. However, higher temperatures within the optimal range for each species stimulate consumption, optimize digestion, and improve growth performance (Neuheimer et al., 2011; Volkoff and Rønnestad 2020).

Therefore, it is interesting to understand whether a higher temperature, but within the limits of thermal comfort, promotes higher tilapia masculinization rates in the BFT system with zero exchange, especially when combined with lower MT concentrations.

In order to reduce the environmental impact and find the lowest masculinizing concentration possible, this study evaluated the masculinization rate of Nile tilapia in the biofloc system (BFT) with zero water exchange. For this experiment, MT concentrations were evaluated below the lowest masculinization rate (30 mg · Kg^{-1} of feed and 99% masculinization rate) obtained by Ramírez et al (2024) in a study with masculinization of tilapia in the same system. Furthermore, it was verified how the increase in temperature, within the thermal comfort range mentioned by Nivelle et al (2019), could interfere with water quality, growth performance, the masculinization process, the amount of hormone used and the generation of effluents containing MT.

4.3. Material and methods

4.3.1 Experimental design

The experiment was conducted at the Aquaculture Laboratory (LAQUA) of the Veterinary School of the Federal University of Minas Gerais (UFMG). All procedures were previously reviewed and approved by the Counsel of ethical practices in animals of the Federal University of Minas Gerais (CEUA) under protocol number 66/2023.

For the experiment, Nile tilapia larvae (11. 57 ± 0.46 mg and 0.93 ± 0.03 cm) were used from spawning and hatching, on the same day, of 12 females (average weight of 766.67 ± 96.98 g), to avoid family × treatment interaction effects. The breeding scheme that gave rise to the spawning's included broodstock of the Chitralada lineage (90 females and 72 males distributed in 9 breeding hapas, in the proportion of 10 females and 8 males each), from the ninth generation of the genetic improvement program for body weight increase (more details are available in Cavatti Neto et al., 2023) from the NGT-Aqua (Nutrition, Genetics and Technology for Aquaculture) research group, belonging to the Aquaculture Laboratory/ UFMG. After the reproduction period (7 days), the females that reproduced were separated individually into incubation hapas.

Soon after incubation and yolk sac absorption the larvae of 12 females that were at the same stage of development were transferred to 30 polyethylene tanks (50 L useful volume), filled with matured biofloc formed previously in other tanks where adult fish have been stocked (25 kg of adult fish · m^{-3}) through the entire year, using the addition of cane sugar as a carbon source to maintain a carbon: nitrogen ratio close to 6: 1, important for heterotrophic bacteria to develop and remove ammonia from the water, together with nitrifiers bacteria (total ammonia nitrogen 0.06 ± 0.03 mg · L^{-1}; nitrite 0.85 ± 0.08 mg · L^{-1} and nitrate 71.93 ± 14.07 mg · L^{-1}). A total of 3.600 larvae were distributed in the tanks (120 larvae · $tank^{-1}$) and it was ensured that each tank of each treatment received the same number of larvae from each of the 12 females. The experimental design was completely randomized, with treatments resulting from the combination of 2 cultivation temperatures (25 and 28 °C) and 5 levels of methyltestosterone (MT) in the diet (0, 10, 20, 30 and 40 mg · Kg^{-1} of feed) for 28 days.

To maintain constant experimental temperatures, the tanks were installed in agricultural greenhouses, and each one received 100-Watt thermostats. Aeration was

maintained by radial blowers connected to microporous hoses installed in each tank to maintain adequate oxygen levels for the species. In addition, during the experiment, were added 75 g of limestone powder in each tank, as a carbonates source, to maintain alkalinity at levels suitable for nitrifying bacteria and the pH close to neutrality.

An initial biometry was conducted with 20 animals, randomly removed from each experimental unit, establishing the initial stocking density of 2 larvae · L^{-1} (n = 100 larvae · $tank^{-1}$). The 20 larvae were weighed together on an analytical balance (©Marte Científica, Brazil), with a precision of 0.001 g. Then, the larvae were euthanized with eugenol (180 mg · L^{-1} for 10 min.) to have their length measured.

The Nile tilapia larvae were fed with a commercial ration (Propescado-Nutriave Foods) containing 55% crude protein, 12% moisture, 10% ether extract and 15% ashes, enriched with MT. Each experimental diet received the masculinizing hormone using 99.5% P.A ethyl alcohol as a vehicle for 17α-methyltestosterone. The solution was previously prepared by weighing the hormone (each hormonal treatment with its specific weighing) and diluting it in 200 mL of ethyl alcohol. After preparing the solution, the liquid was distributed over the diet using a 500 ml spray bottle. Then, the diet was stored away from light to evaporate the alcohol. The diets were identified and stored in a freezer at -20 °C, protected from light, to preserve MT throughout the experiment.

In the period after masculinization, the fish received a hormone-free diet until they reached an adequate size for sex identification analysis using the aceto-carmine technique (Guerrero and Shelton, 1974).

The larvae were fed five times a day at a feeding rate of 30%, 25%, 20% and 15% of body weight in the first, second, third and fourth week, respectively, according to Costa e Silva et al. (2022) and Ramirez et al. (2024). Feeding correction was guided by weekly biometrics, conducted with samples from 20 larvae per tank, estimating a probable weight gain in the following week based on the weight gain of the previous week and a mortality of 3% for each week.

Treatments at 28 °C had a higher feed consumption than treatments at 25 °C, therefore, the amount of hormone per larvae was different between these two groups, as calculated below. Considering the treatment of 40 mg of MT · Kg^{-1} of feed, as a reference, at 25 °C treatments, the amounts of feed and hormone offered to each larvae during the

experimental period were, respectively, 236.14 mg of feed and 9,445.66 ng of MT for each larvae. In contrast, at 28° C, 775.72 mg of feed were used and 31,028.76 ng of MT for each larva.

4.3.2 Water quality

During the entire experimental period, there was no water renewal or solids removal from the tanks, with the volume of water lost through evaporation being supplemented, daily, with clear water from an artesian well.

Temperature, pH, oxygen, and salinity measurements were taken three times a week in the tanks of all treatments. To monitor and ensure that thermal parameters were maintained at 25 °C and 28 °C according to the treatment, Exbom digital thermo-hygrometers were used, where the minimum and maximum temperatures were evaluated daily. With these measures, thermostats could be adjusted to the desired temperature. Total ammonia nitrogen (TAN), non-ionized ammonia (NH_3) and nitrite (NO_2^-) were measured twice a week. Alkalinity and settleable solids (SS) analyzes were conducted once a week, and nitrate (NO_3^-), total nitrogen (N), total suspended solids (TSS) once, at the end of the experiment.

Dissolved oxygen levels ($mg \cdot L^{-1}$) were measured using an AT 155 oximeter (Alfakit®, Florianópolis, Santa Catarina, Brazil); and pH, salinity ($g \cdot L^{-1}$) and temperature (ºC) were monitored using a multiparameter probe (Hanna®, Barueri, São Paulo, Brazil). The levels of total ammonia nitrogen (TAN) and nitrite (NO_2^-) were measured according to the methodologies established by UNESCO (1983) and Bendschneider and Robinson (1952), respectively, while the nitrate (NO_3) was quantified using the method described by Monteiro et al. (2003). Alkalinity was measured using the methodologies proposed by APHA (1998). Settable solids (SS) were quantified using Imhoff cones (Avnimelech, 2006). Total suspended solids (TSS) were measured after collecting 20 ml of water, with subsequent filtration through GF50-A glass fiber filters, which were then dried and weighed to quantify the retained material.

4.3.3 Growth performance, survival, and masculinization rate

The growth performance of tilapia was evaluated by the final body weight (BWf), final body length (BLf), specific growth rate (SGR), Fulton condition factor (CF) and survival (S), at the 29th day, in the morning, before the first feeding of the day, as follows:

final body weight (BWf) = mean of the mass (g) of 20 larvae randomly picked per experimental unit;

final body length (BLf) = mean of body length (cm) of 20 larvae randomly picked per experimental unit;

specific growth rate (SGR) = 100 × (log (final body weight) – log (initial body weight)) / days of experiment;

condition factor (CF) = final body weight (g) × total length^{-3} (cm) x 100;

survival (S) = (final number of individuals / initial number of individuals) x 100;

feed consumption = amount of feed offered (g);

biomass = BWf (g) × final number of individuals;

productivity = biomass (g) / 50 L = kg×m^{-3}

feed conversion ratio (C.A.) = feed consumption / biomass

After a period of 28 days of experiment, the fingerlings from each treatment were removed, as they reached the minimum size of 3.5 cm for sexing, to verify the effect of MT concentrations on masculinization. For this analysis, tilapias were euthanized via eugenol-induced overdose (180 mg · L^{-1} for 10 min.) (Vidal et al., 2008). The fingerlings were fixed in Bouin's liquid for 24 hours and dehydrated in 70% ethyl alcohol. Subsequently, their gonads were removed and analyzed under an optical microscope, using 40x objectives, using the aceto-carmine technique (Guerrero and Shelton, 1974).

4.3.4 MT residues in the water

For each treatment, three 40.0 mL water samples were collected 2 hours after the last feeding with hormone-containing food and for the subsequent 10 hours, placed in 50 mL plastic centrifuge tubes, and stored at -20 °C in the dark until analysis. MT analysis in water was first validated in-house so that water samples from the tanks could be evaluated, following the procedures below:

Chemicals and reagents

Acetonitrile, methanol, and ethanol HPLC grade were purchased from Sigma Aldrich (St. Louis, MO, USA). Water was purified in-house with a Milli-Q water purification system (18.2 Ω, Millipore Co., MA, USA). All other chemicals were higher than analytical grade and obtained from commercial sources. Methyltestosterone (MT) standard were purchased from Active Pharmaceutica Ltda (Palhoça, SC, Brazil) with purity ≥ 99%. The standard stock solution was prepared in methanol at a concentration of 1000 $\mu g \cdot mL^{-1}$. The working solutions were prepared through appropriate dilutions of the stock solutions. All the solutions were stored at -20 °C for subsequent use. Solid-phase extraction Discovery SPE DSC-18 tubes (500 mg, 6 mL) were purchased from Aldrich Chemical (Sigma–Aldrich, St. Louis, MO).

Sample preparation

Blank biofloc water samples were spiked with MT to validate the analytical method and were then processed following the same procedure as the treatment samples. For each treatment, three 40.0 mL water samples were collected, placed in 50 mL plastic centrifuge tubes, and stored at 4 °C in the dark until analysis. The samples were then centrifuged at 6000 G for 10 minutes to allow sediment precipitation, followed by extraction and clean-up using solid-phase extraction (SPE) cartridges. The SPE cartridges were conditioned by passing 5.0 mL of acetonitrile through them, followed by 5.0 mL of Milli-Q water. All samples were extracted by passing them through the SPE cartridges at a flow rate of 2.0 mL/min. A washing step with Milli-Q water was performed before elution with 5.0 mL of HPLC-grade ethanol. The eluate was evaporated to dryness at 37-40 °C, and the residue was re-dissolved in 1000 µL of mobile phase. The resulting extracts were used for chromatographic analysis.

Preparation of calibration standards

Appropriate amounts of stock/working solution were spiked into blank matrix extracts to prepare the series of matrix matched calibration standards of 50, 100 and 200 $\mu g \cdot L^{-1}$.

Instrument and HPLC conditions

High performance liquid chromatography (HPLC) analyses were performed using an JASCO LC-2000Plus HPLC System (Jasco International CO., LTD., Hachioji, Tokyo, Japan) equipped with a quaternary gradient pump (PU-2089S), 4-line degasser, autosampler (AS 2059), column oven (CO-2065) and UV/VIS detector (UV-2075). Chromatographic separations were performed in reverse phase using a C18 column (150 x 4.6mm, 5μm particle size) (Supelcosil™ LC-ABZ, Supelco, Bellefonte, PA, USA). The mobile phase used was acetonitrile:methanol:water (50:10:40 v/v/v) in isocratic mode. The injected volume was 100 μL, the flow was 1.0 mL per minute, and the column temperature was set to 25 °C. MT was detected at 245nm using a UV detector. Peak area values were plotted against concentration and calibration curves for MT were constructed using least squares method for linear regression. ChromNAV 2.0 software was used for data acquisition and data processing. Each sample was analyzed in triplicate.

Parameters for Method Validation

Selectivity refers to the capability of an analytical method to distinguish the target analytes from other unrelated substances present in the sample. To evaluate selectivity, six batches of blank biofloc water were analyzed. Chromatograms of the blank samples were compared with those of the spiked samples containing MT, to assess whether the target analyte exhibited the same retention time as any potential interfering compounds present in the sample.

The matrix effect is the influence that other components in the sample, other than the analyte of interest, have on the analytical method. To assess this, a calibration curve was initially established using calibration standards in a solvent (mobile phase). A second calibration curve was then generated using matrix-matched calibration standards. Linearity of both curves was examined through *F*- and *t*-tests, while the matrix effect was evaluated by comparing the parallelism of the response curves. A significance level (α) of 0.05 was applied in all statistical tests.

For MT, a calibration curve was constructed by analyzing biofloc water extract samples spiked with the analyte at various concentrations over the course of three separate days. The peak area data were plotted against the concentration of MT, and calibration

plots were created using weighted least-squares linear regression. The correlation coefficient (r) and coefficient of determination (R^2) were also determined.

The limit of detection (LOD) represents the lowest concentration that produces a signal distinguishable from background noise and was calculated using a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was determined as the lowest concentration that could be reliably quantified, calculated based on a signal-to-noise ratio of 10:1. These values were derived from the analysis of three replicate samples.

Method precision was evaluated through repeatability studies and intra-laboratory reproducibility. Both intra-day and inter-day precision were assessed at three different concentration levels, with six replicates for each level, measured on the same day and across three consecutive days. Precision was expressed as the relative standard deviation (RSD) to quantify the variability of measured concentrations.

Accuracy was evaluated using the spike recovery method. MT was spiked into the matrix at three concentration levels, with six replicates for each concentration. After sample preparation, the spiked material was analyzed from start to finish, including chromatographic determination. The recovery (%) of the method was determined by comparing the amount of analyte found with the amount added to the sample.

4.3.5 Statistical evaluation

For all variables (except for masculinization proportions), linear regression models were adjusted. When the assumptions of normality and homogeneity of variances were not met, according to the Shapiro-Wilk and Bartlett tests, respectively, log data transformation and/or weighted least square method were used. Differences were considered significant when $p < .05$. The chi-square test with Bonferroni correction was used to compare masculinization proportions. Statistical analyzes were performed using the InfoStat program (Di Rienzo et al., 2015) and R software (R Core Team, 2021).

4.4 Results

4.4.1 Water quality

Except for the temperature, which was below the recommended level due to the tested factor itself, and the suspended solids, which were slightly below ideal due to care not to over-agitate the water due to the size of the larvae, all other parameters were within the reference values for the species. The salinity of this experiment showed values between 0.12 and 0.14 $g \cdot L^{-1}$, which are close to 0.00 $g \cdot L^{-1}$, ideal for masculinization in BFT (Do Valle et al., 2023).

The average temperature values were consistent with the treatments applied. Throughout the experimental period, the 25 °C treatment had a mean of 24.79 °C and there was a mean significant difference of +3.73 °C ($p < 0.0001$) between this value and the average of the 28 °C treatment (Table 4), regardless the MT concentration and without the interaction with this factor, such as it was expected due to temperature control with heaters thermostats to maintain the two different temperatures defined by the experimental design.

Table 4. Means ± standard deviation of water quality variables per temperature (T, 25 °C or 28 °C), in combination with four different concentrations of 17α-methyltestosterone (MT) in the diet (0, 10, 20, 30 and 40 mg · Kg^{-1} of feed) and p values associated with sources of variation and their interaction (T × MT) from Nile tilapia masculinization in a biofloc system.

Variables	T	MT					p-values			Regression models; adjusted R^2	[1]Reference values
		0	10	20	30	40	T	MT	T*MT		
Temperature (°C)	25°C	24.76 ± .06	24.68± .12	24.92± .15	25.30± .22	24.70± .24	<.0001	.408	1.0	Y= 24.79 + 3.73*T; adjR^2= .98	27-32[a]
	28°C	28.34 ± .14	28.62± .34	28.70± .23	28.77± .18	28.56± .50					
pH	25°C	6.99 ± .08	6.98± .03	6.94± .05	7.01± .02	7.01± .09	.0032	.5245	.6033	Y= 6.97 - .14*T; adjR^2= .57	6-9[b]
	28°C	6.87 ± .09	6.77± .01	6.86± .10	6.85± .06	6.83± .06					
Oxygen ($mg.L^{-1}$)	25°C	6.99 ± .15	6.98± .14	6.89± .05	6.87± .20	7.01± .16	.0372	.7721	.6318	Y= 6.96 + .22*T; adjR^2= .22	>4[b]
	28°C	7.14 ± .10	7.23± .20	7.22± .23	7.00± .03	7.10± .21					
Settleable solids ($mL{\cdot}L^{-1}$)	25°C	.93 ± .20	1.02± .65	1.39± .65	2.39± .38	2.02± .77	<.0001	.084	.3819	Y= .84 + 5.65*T; adjR^2= .90	25-50[c]
	28°C	6.14 ± .25	7.12± .40	8.42± .70	7.91±1.70	8.99±3.55					
Total suspended solids ($mg{\cdot}L^{-1}$)	25°C	1.50 ± .61	.77± .55	4.33±1.80	4.67±2.76	1.80± .40	<.001	.6167	.2536	Y= 1.71 + 13.52*T; adjR^2= .54	<1000[d]
	28°C	16.0 ±4.85	12.59±9.89	14.10±11.36	12.23±2.04	11.09±4.43					
Alkalinity (mg de $CaCO_3{\cdot}L^{-1}$)	25°C	92.33 ±3.06	84.0±8.72	75.67±12.42	95.67±11.85	93.0±6.93	.0026	.4974	.4079	Y= 85.53 – 21.6*T; adjR^2= .43	>20[b]
	28°C	70.0 ±7.94	56.33±4.16	76.33±11.93	73.0±6.08	79.33±4.04					
TAN ($mg{\cdot}L^{-1}$)	25°C	.06 ± .03	.02± .01	.01± .01	.002± .002	.01± .02	.1711	.0122	.0320	Log(Y) = -3.31 – .08*MT + .09*T*MT; adjR^2= .15	<1[d]
	28°C	.02 ± .01	.03± .04	.005± .005	.03± .03	.04± .03					
Nitrite (NO_2) ($mg{\cdot}L^{-1}$)	25°C	1.10 ± .33	.86± .29	1.24± .27	1.39± .69	1.72± .48	.5513	.0086	.0043	Y= .94 + .017*MT - .026*T*MT; adjR^2= .41	<8[a]
	28°C	1.12 ± .27	.92± .28	.84± .15	.81± .18	.72± .13					
Nitrate (NO_3) ($mg{\cdot}L^{-1}$)	25°C	76.23 ± 27.41	44.18±26.29	45.42±24.80	72.61±38.58	51.39±21.25	<.001	.8198	.9519	Log(Y) = 3.97 + 1.38*T; adjR^2= .59	<500[e]
	28°C	144.28±27.41	378.77±163.18	207.85±140.49	214.63±24.32	167.84±84.14					

Letter "T" is an indicator variable that receives the value one (1) if the temperature treatment was 28 °C and the value zero (0) if the temperature treatment was 25 °C.

[1]Reference values:
a El-Sayed (2019).
b Wedemeyer (1996).
c Hargreaves (2013).
d Avnimelech (2009).

e Monsees et al. (2017).

The pH and dissolved oxygen levels varied slightly between treatments. The 25 °C treatment had a mean of 6.97 and 6.96 $mg \cdot L^{-1}$, for the respective water quality variables. The 28 °C treatment had a decrease of – 0.14 ($p = 0.0032$) and an increase of + 0.22 $mg \cdot L^{-1}$, respectively to the two variables and regardless the MT concentration and the interaction with temperature.

The settleable solids and total suspended solids had a significant increase due to the temperature. At 25 °C, the mean value was 0.84 $mL \cdot L^{-1}$ and 1.71 $mg \cdot L^{-1}$, respectively for these water quality variables. The 28 °C treatment had an increase of + 5.65 $mL \cdot L^{-1}$ ($p < 0.0001$) and + 13.52 $mg \cdot L^{-1}$ ($p < 0.001$), respectively to the two variables and regardless the MT concentration and the interaction with temperature. However, the mean alkalinity value decreased with increasing temperature. At 25 °C, the mean value was 85.53 mg $CaCO_3 \cdot L^{-1}$ and at 28 °C, there was a decrease of – 21.6 mg $CaCO_3 \cdot L^{-1}$ ($p = 0.0026$), also without MT concentration effect and its interaction with temperature.

Total ammonia and nitrite showed low concentrations for all treatments. For total ammonia there was a slight reduction by the increase of MT concentration ($p = 0.0122$) and an increase by the T × MT interaction ($p = 0.0320$). At the control treatments (0 mg of $MT \cdot Kg^{-1}$ of feed) the mean value was 0.03 $mg \cdot L^{-1}$. At 25 °C, values ranged from 0.002 ± 0.002 $mg \cdot L^{-1}$ (MT = 30 $mg \cdot Kg^{-1}$) to 0.06 ± 0.03 $mg \cdot L^{-1}$ (MT = 0 $mg \cdot Kg^{-1}$). At 28 °C, concentrations varied between 0.005 ± 0.005 $mg \cdot L^{-1}$ (MT = 20 $mg \cdot Kg^{-1}$) and 0.04 ± 0.03 $mg \cdot L^{-1}$ (MT = 40 $mg \cdot Kg^{-1}$). For nitrite, there was a slight increase by the increase of MT concentration ($p = 0.0086$) and a decrease by the T × MT interaction ($p = 0.0043$). At the control treatments (0 mg of $MT \cdot Kg^{-1}$ of feed) the mean value was 0.94 $mg \cdot L^{-1}$. At 25 °C, values varied between 0.86 ± 0.29 $mg \cdot L^{-1}$ (MT = 10 $mg \cdot Kg^{-1}$) and 1.72 ± 0.48 $mg \cdot L^{-1}$ (MT = 40 $mg \cdot kg^{-1}$). At 28 °C, values ranged between 0.72 ± 0.13 $mg \cdot L^{-1}$ (MT = 40 $mg \cdot Kg^{-1}$) and 1.12 ± 0.27 $mg \cdot L^{-1}$ (MT = 0 $mg \cdot kg^{-1}$).

Nitrate and total nitrogen were positively strongly influenced by temperature ($p < 0.001$), presented mean values at 25 °C of 52.98 and 12.3 $mg \cdot L^{-1}$, and at 28 °C of 210.6 and 47.94 $mg \cdot L^{-1}$, respectively.

4.4.2 Growth performance, survival, and masculinization rate

The initial body weight and length of the fry did not show significant differences between all treatments, with a mean value of 11.64 mg and 0.92 cm (Table 5), showing a good initial distribution of the larvae in the tanks.

Table 5. Means ± standard deviation of performance variables per temperature (T, 25 °C or 28 °C), in combination with four different concentrations of 17α-methyltestosterone (MT) in the diet (0, 10, 20, 30 and 40 mg · Kg^{-1} of feed) and p values associated with sources of variation and their interaction (T × MT) from Nile tilapia masculinization in a biofloc system.

Variables	T	MT					p-values			Regression models; adjusted R^2
		0	10	20	30	40	T	MT	T*MT	
Initial body weight (mg)	25°C	11.91± .48	11.18± .14	11.57±0.25	11.78±0.52	11.45±0.34	.520	.643	.793	Y= 11.64
	28°C	11.44± .27	11.53± .30	11.10±0.07	11.70±0.07	11.15±0.10				
Final body weight (mg)	25°C	319.60±147.09	236.50±48.01	248.79±10.45	311.99±27.76	293.69±4.16	<.0001	.891	.129	Y= 277.38 + 475.40*T; adjR^2= .90
	28°C	772.23±111.34	803.10±126.32	752.44±126.26	739.27±295.60	932.96±141.47				
Initial body length (cm)	25°C	.90± .05	.95± .01	.94± .01	.94± .03	.94± .04	.713	.269	.297	Y= .92
	28°C	.93± .03	.91± .02	.92± .05	.95± .03	.90± .03				
Final body length (cm)	25°C	2.39± .22	2.30± .10	2.29± .03	2.46± .05	2.37± .01	<.0001	.714	.338	Y= 2.34 + .99*T; adjR^2= .91
	28°C	3.32± .25	3.44± .32	3.38± .22	3.33± .43	3.57± .20				
Specific growth rate (%·day^{-1})	25°C	11.52±1.48	10.85± .77	10.96± .23	11.69± .45	11.59± .06	<.0001	.566	.855	Y= 11.12 + 3.76*T; adjR^2= .80
	28°C	15.02± .46	15.48± .81	14.17±1.10	14.49±1.55	15.79± .55				
Condition Factor	25°C	2.23± .40	1.94± .15	2.08± .17	2.10± .06	2.22± .06	.893	.851	.391	Y= 2.09
	28°C	2.13± .25	2.19± .19	1.85± .20	1.92± .14	2.05± .04				
Survival (%)	25°C	97.00±4.24	95.00±2.83	93.67±3.79	94.50±7.78	94.33±1.53	.126	.639	.629	Y= 95.89
	28°C	89.67±5.69	91.00±3.00	90.67±3.06	88.33±13.20	92.00±6.08				
Productivity ($Kg·m^{-3}$)	25°C	.68± .34	.42± .14	.47± .03	.55± .09	.55± .01	<.0001	.947	.127	Y= .53 + .81*T; adjR^2= .88
	28°C	1.38± .12	1.44± .26	1.37± .29	1.46± .04	1.72± .30				
Feed consumption (g)	25°C	41.48±1.17	45.68± .72	43.75± .74	43.69±1.06	46.11±1.12	<.0001	.348	.345	Y= 42.37 + 80.02*T; adjR^2= .99
	28°C	117.91±7.81	131.51± .59	123.31±1.49	127.02± .80	129.64± .71				
Feed conversion ratio	25°C	1.37± .68	1.84± .03	1.88± .11	1.61± .25	1.66± .003	.523	.663	.456	Y= 1.63
	28°C	1.72± .22	1.68± .40	1.85± .39	1.74± .03	1.54± .25				

Final body weight and length were higher at 28 °C, presenting an average increase of + 475.40 mg and .99 cm ($p < 0.0001$) in relation to 25 °C treatment. The mean values at 25 °C were 277.38 mg and 2.34 cm and at 28 °C were 752.78 mg and 3.33 cm, respectively.

Following the final body weight and length, the specific growth rate of 28 °C treatments were 3.76 %·day^{-1} higher than 25 °C ($p < 0.0001$), presented mean value of 11.2 %·day^{-1} at 25 °C and 14.96 %·day^{-1} at 28 °C, regardless MT concentration effect and its interaction with temperature. However, condition factor and survival values did not show significant differences between treatments, with average values of 2.09 and 95.89%, respectively (Table 2).

The increase in the final body weight at 28 °C and the equivalence of survival led to a higher productivity (+ 0.81 Kg·m^{-3}, p value < 0.0001) and higher feed consumption (+ 80.02 g, p value < 0.0001) at the treatments this temperature was applied. However, feed conversion ratio did not show significant differences between treatments, with an average value of 1.63 (Table 2).

There were significant differences in the proportions of males, between the control and the other MT concentrations, regardless of the temperatures ($p < .05$) (Fig. 1- C). The proportion of non-males (females and intersex) in the control, without the presence of hormones in the feed, was 31.40% and 35.10% at 25 °C and 28 °C, respectively. There were no differences between the proportion of males of MT concentration treatments ranging from 10 to 40 mg of MT · Kg^{-1} of feed, at 25 °C ($p > .05$) and no difference between them and the respective treatments at 28 °C ($p > .05$). However, there were differences between the proportion of males of 10 and 20 mg of MT · Kg^{-1} of feed (97% and 96.9%, respectively), and 40 mg of MT · Kg^{-1} of feed (100%) at 28 °C ($p > .05$).

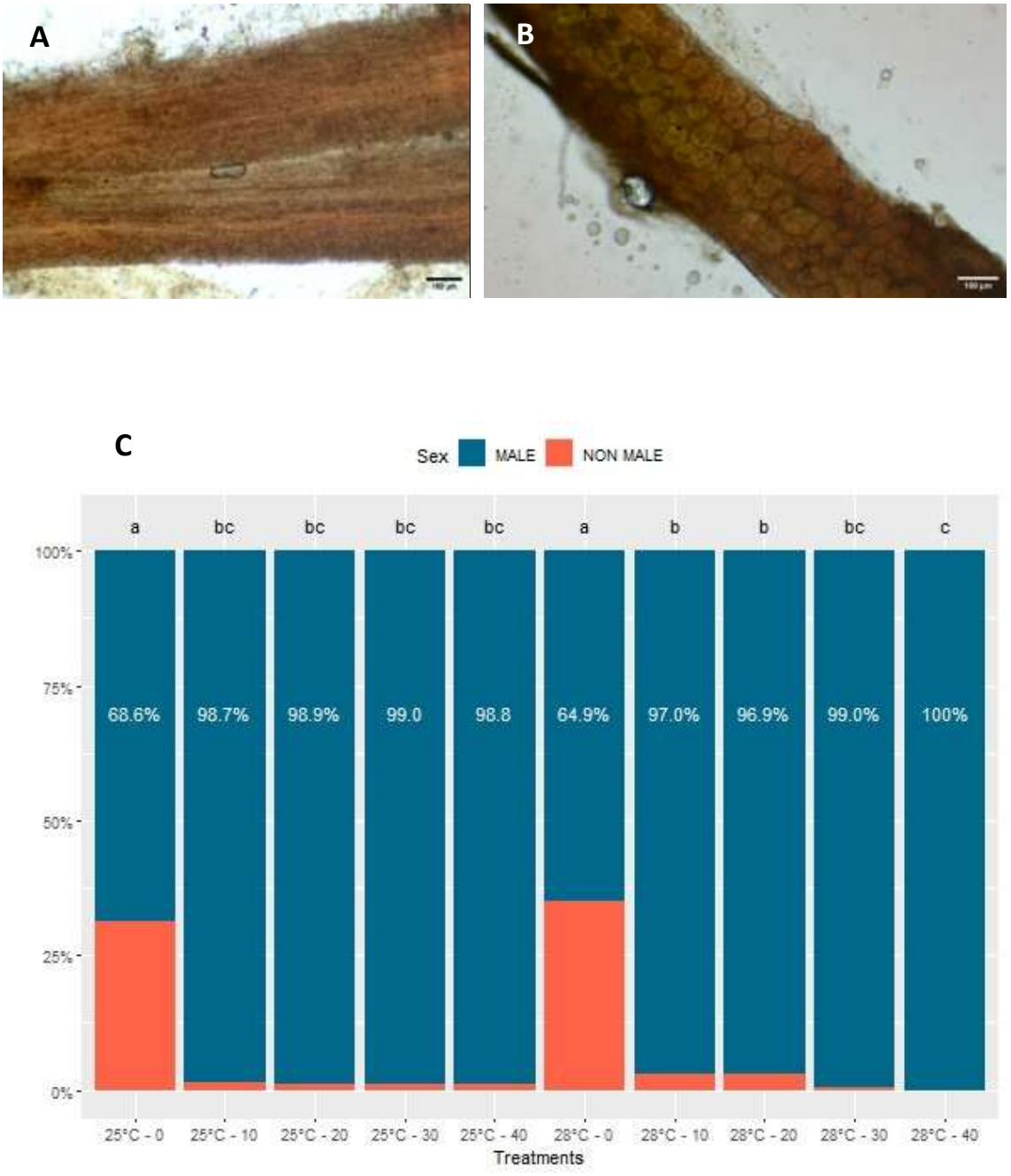

Figure 3. Results of sexing analysis of Nile tilapia fed diets containing different concentrations of 17α-methyltestosterone during the masculinization phase. A) Microscopic image of a male gonad subjected to the aceto-carmine technique from an animal in the control group of Nile tilapia produced using biofloc technology (BFT). B) Microscopic image of female gonad subjected to the aceto-carmine technique from a Nile tilapia control animal produced using biofloc technology (BFT). C) Proportion of males and non-males (females and intersex) of Nile tilapia during 28 days of masculinization under different concentrations of 17α-methyltestosterone in the diet (0, 10, 20, 30 and 40 mg · Kg^{-1} of feed) at 25 °C and 28 °C of temperature in biofloc technology (BFT).

4.4.3 MT residues in the water

The results of validation and the analysis of the water samples are presented below.

Selectivity

To assess selectivity, the chromatograms of the blank sample extracts were compared with those of the fortified samples. The absence of signals, considering the respective retention times and signal-to-noise ratios, indicated that no matrix interference occurred at the same retention time as the target analyte (MT). This confirmed the excellent selectivity of the method (Ribani, 2004).

Precision and Accuracy

The precision and accuracy of the method were evaluated by assessing both inter- and intra-day variability for MT at four different concentration levels. The results, shown in Table 3, revealed precision values expressed as relative standard deviation (RSD) ranging from 6.282% to 8.813%. These values were consistent with the guidelines provided by INMETRO (2020), remaining within the acceptable limit of 20%. Accuracy, expressed as recovery percentage, ranged from 70.79% to 99.88%. These recovery values were within the acceptable range of 70-120% as stipulated by Ribani (2004) and INMETRO (2020). These promising results demonstrate that the method developed is highly dependable for the analysis of MT in biofloc water, showing both excellent precision and accuracy.

Table 6. Intra-day and inter-day precision and accuracy results of methyltestosterone in biofloc water at four different levels ($\bar{x}$±SD).

Methyltestosterone	Intra-day (*n* = 6)		Inter-day (*n* = 6)	
Spiked concentration (μg·L^{-1})	Measured concentration (μg·L^{-1})	Precision (RSD, %)	Measured concentration (μg·L^{-1})	Precision (RSD, %)
50	35.665 ± 2.967	8.321	35.393 ± 3.119	8.813
100	101.065 ± 7.595	7.515	99.880 ± 6.986	6.995
200	152.932 ± 11.964	7.823	153.630 ± 9.651	6.282

Calibration Curves, LOQ, and LOD

The analytical calibration curve for MT demonstrated excellent linearity. The linear regression equation was y=1E+06x+1267.3, resulting in a correlation coefficient (r) of 0.999 and a determination coefficient (R^2) of 0.998. The curve was constructed using ten different concentration levels of MT (25, 50, 100, 200, 400, 600, 1000, 1500, 2000, 2500 μg·L^{-1}), meeting the requirements set by the International Conference on Harmonization (ICH), the National Health Surveillance Agency (ANVISA), and the Group of Pesticide Residue Analysts (GPRA), which recommend a calibration curve with at least five concentration levels (ICH, 1994; ANVISA, 2003). This result complies with Brazilian regulations, which specify that the coefficient of determination (R^2) should be between 0.99 and 0.90 (ANVISA, 2003). The limit of quantification (LOQ) of the calibration curve was determined to be 25 μg·L^{-1} based on a signal-to-noise ratio (S/N) of 10, while the limit of detection (LOD) was calculated at 10 μg·L^{-1}, based on an S/N of 3. These results demonstrate that the method is suitable for quantifying MT in biofloc water.

Matrix Effect

In the matrix effect evaluation, the analytical curves of the solvent standards were compared with those of the matrix-matched standards. No significant statistical differences were found between the two sets of curves. As a result, the method was validated using calibration curves constructed in the solvent, as this approach yielded comparable results to those obtained in the matrix.

Determination of MT in biofloc water samples

None of the biofloc water samples showed detectable residues of MT at 2 hours after the last feeding with hormone-containing food.

4.5 Discussion

The hormonal treatment with MT in masculinization of Nile tilapia is used in productions spread in the whole world to obtaining uniformity in harvesting, higher growth rates, and avoiding overpopulation, which causes harm to fish farmers (Singh, 2013; Mlalila et al., 2015; Baroiller and D'Cotta, 2018). However, this practice, generally carried out in tilapia hatcheries, is not environmentally friendly due to periodic water changes, which contaminate natural bodies of water with MT.

The masculinization process in BFT using zero water exchange has become an alternative to the traditional method in tilapia hatcheries, as it allows the reduction and complete elimination of MT residues in the water, as demonstrated by Ramírez et al (2024) in the masculinization of Nile tilapia in BFT, testing MT concentrations (00, 30, 40, 50 and 60 mg of MT · Kg^{-1} of feed) lower than those commonly used in nurseries (60 mg of MT · Kg^{-1} of feed). The authors concluded that using the lowest concentration evaluated (30 mg of MT · Kg^{-1} of feed) it was possible to achieve masculinization rate above 99%, considerably high and satisfactory for production. These results signaled that tilapia masculinization can be conducted in a more environmentally responsible way, using a zero-water exchange BFT, but also indicated that even lower concentrations should be evaluated. Furthermore, one fact in particular caught attention in the study by Ramírez et al. (2024), and gave rise to a new question: try to understand if the temperature used, 28 °C, have influenced the masculinization rates, because control treatment, whose post-larvae received hormone-free feed, presented a number of males notably higher than what would correspond to half of the animals, as expected.

Therefore, for the present study two hypotheses were evaluated. Whether it is possible to achieve high masculinization rates by further reducing the MT concentration in the feed and whether a higher temperature could contribute to this increase in a masculinization protocol of Nile tilapia larvae in a zero-water exchange BFT. To

elucidate these two issues, MT concentrations of 00, 10, 20, 30 and 40 mg · Kg^{-1} of feed were evaluated using two different temperatures, 25 °C and 28 °C.

4.5.1 Water quality

Water quality variables were within reference values for the species. The temperature was within the proposed experimental values. Although there were variations in oxygen concentrations depending on temperature, these were minimal and unable to promote any type of change in the other variables. Higher temperatures promote increased consumption due to accelerated metabolism (Volkoff and Rønnestad, 2020). Because of greater feed consumption at 28 °C when compared with 25 °C, there is an increase in excretion and formation of settleable solids, total suspended solids, and nitrate as well. The subtle and expressive reduction in pH and alkalinity, respectively, and a low ammonia and nitrite and an increase in nitrate at 28 °C temperature is an indicative of a well-functioning nitrification process.

The increases in nitrite concentrations due to the increase in MT were minimal, lower than the reference value for the species (Table 1), and unable to cause any type of damage to the growth performance of the fish. Despite being increased at higher temperature, the concentration of nitrate in the water was well below the limit for the species, causing no changes in growth or even survival (Table 2).

These results demonstrate the important role of the BFT system in maintaining water quality parameters. Even though there was a greater feed intake at a temperature of 28 °C, it was still not enough to harm the environmental conditions. On the one hand, this is due to the proportional incorporation of nitrogen in the muscle composition of the fish at the highest temperature, since there was no variation between feed conversion between 25 °C and 28 °C, and the system's ability to eliminate toxic nitrogenous components.

4.5.2 Growth performance, survival, and masculinization rate

Growth performance did not vary because of increasing MT concentration in the feed, corroborating the results of Ramírez et al. (2024), who also found no differences even when using higher concentrations (50 and 60 mg of MT · Kg^{-1} of feed). However,

according to literature changes in growth performance may occur due to the use of MT in different contexts, and due to interference from factors such as the species, the concentration of MT used in the feed, and the time of hormonal treatment. Karsli (2021), in a study with the ornamental cichlid *Aulonocara nyassae*, at 5 months of age, observed an increase in weight gain as the higher the MT concentration was, thus, using 00, 50, and 100 mg of MT $\cdot$ Kg^{-1} of feed, found, after 60 days of experiment, mass gains of 116.90 g; 170.42 g, and 214.08 g, respectively. Factors such as feeding rate, as well as the production system used, can also determine the anabolic condition of MT in fish; however, in the case of tilapia masculinization in BFT, this effect has not been observed (Costa and Silva, 2022; Ramírez et al., 2024).

Water temperature and its variations directly influence the regulation of the metabolic rate and physiology of fish, with direct impacts on growth performance (Little et al., 2020; Schulte, 2011). The increase in consumption and growth variables in fish subjected to higher temperatures, as long as they are within the optimal range for each species, is widely reported in studies. In this study, although the growth performance and survival variables were not affected by the hormone concentrations tested, differences were observed between specific growth rate, final body weight and length of larvae produced at temperature of 25 °C and 28 °C, with the greatest development at highest temperature. It is possible to observe that treatments at 28° C the final body weight of tilapia fingerlings was 2.7 times higher than reared at 25 °C. A study carried out by Mourad et al. (2018) with tambaqui (*Colossoma macropomum*), pacu (*Piaractus mesopotamicus*) and their hybrids tambacu and paqui, found that body weight, length, width and height have a positive correlation when temperatures vary between 26 °C and 29 °C, and lower temperature values result in the reduction of growth performance. In the experiment carried out by Azaza et al. (2008), 20-day-old tilapia were subjected to 22, 26, 30 and 34 °C to evaluate their growth performance and the fishes exposed to extreme temperatures (22 and 34 °C) had worse food utilization, lower growth and average final weight than at 26 °C and 30 °C, corroborating our results, where the lower temperature presented lower consumption and lower development. In a second experiment, the same authors tested temperatures 19, 32, 34 and 36.5 °C in larvae during the first 28 days of life and found the lowest survival rates (60% and 75%) at extreme temperatures (19 °C

and 36.5 °C, respectively), and the highest survival rates (92% and 93%) at intermediate temperatures (32 °C and 34 °C, respectively), indicating that the further the temperatures move away from the which is considered optimal, the lower the growth and the higher the mortality. Although in this actual experiment the highest temperature is within the optimal range (27°-30 °C) for growth in Nile tilapia cited by Nivelle et al. (2019), the lowest temperature is two degrees below this minimum limit, however, no variations in survival were noticed between the 25 °C and 28 °C temperatures.

With the aim of reducing the concentration of MT in the masculinization of Nile tilapia, Ramírez et al. (2024) obtained rates greater than 99% of males using 30 mg · Kg^{-1} of feed, without causing changes in water quality or growth variables, which means a significant reduction in environmental impacts. The authors also suggested that lower concentrations could be sufficient to obtain good masculinization rates, just as higher temperatures could allow an even greater reduction in MT concentration in the masculinization process. In the present study, it was possible to further reduce this concentration and confirm the environmentally friendly nature of masculinization in BFT. In this experiment, using MT concentrations of up to one-sixth (10 mg · Kg^{-1} of feed) of that commonly used in tilapia hatcheries (60 mg · Kg^{-1} of feed), it was possible to achieve masculinization rates greater than 97% and 99% in waters at 28 °C and 25 °C, respectively. There were no differences in masculinization rates between different concentrations of MT within the temperature 25 °C nor between masculinization at 25 °C and 28 °C. However, at 28 °C, there were higher masculinization rates at the concentration 40 mg · Kg^{-1} of feed (100%) than at concentrations 10 and 20 mg · Kg^{-1} of feed (97 and 96.9%, respectively). This small difference can be considered insignificant for a production scenario. It may be related to a greater difficulty in predicting larval growth, which is much greater at a temperature of 28 °C, based on their performance in the previous week. This difficulty may have resulted in a slightly lower feed quantity than necessary to be offered, implying in a smaller amount of MT available to the larvae of the two treatments with lower MT concentration in the diet (10 and 20 mg · Kg^{-1} of feed).

In a study with tilapia masculinization in a recirculation system, Wang et al. (2022) obtained, at a temperature of 28 °C, masculinization rates of approximately 95% and 100% at concentrations of 10 and 20 mg · Kg^{-1} of feed, respectively, corroborating

our results using the same MT concentrations, and opposing the idea that in BFT fish could have lower masculinization rates than in clear water due to consumption of the system's own flocs and reduction of feed containing MT, presented by David-Ruales et al. (2019) and by Costa e Silva et al. (2022). Furthermore, the authors demonstrated that at concentrations below 10 mg · Kg^{-1} of feed, the temperature of 36 °C plays a predominant role in masculinization, which contrasts with our work, in which we did not observe any difference between the control treatments (without MT) at 25 °C and 28 °C, confirming that 28 °C is not a sufficient temperature to promote an increase in masculinization rates. However, the sensitivity to the effect of temperature on masculinization, as well as the high number of males in the treatment that did not receive MT, may be associated with the difference in the lineages of the fish tested.

Despite this, during the 28 days the amount of MT that entered the system was lower at 25 °C, as lower temperatures reduce metabolism, reduce consumption, delay growth, and consequently, reduce the supply of feed containing MT, which makes this procedure even more environmentally sustainable than when using 28 °C temperature. In other words, by performing masculinization with 10 mg · Kg^{-1} of feed at a temperature of 25 °C, it is possible to drastically reduce the amount of MT used in this procedure. On the other hand, although sustainable from an environmental point of view, this may not be true from an economic point of view, as fish masculinized at lower temperatures will take longer to reach commercial weight, which can generate more operational costs during this additional period of time, which in this experiment took two weeks, considering the fish reached the minimum weight of 1 g on average.

Contrary to what was expected, at the same MT concentration, the use of a higher temperature *per se* does not increase the masculinization rate. The explanation may lie in the fact that at 28° C the need for MT for masculinization falls short of what is provided due to rapid growth, and, although the calculation for feed supply considers the mass gain of the previous week, it may still be insufficient for accompany an accelerated metabolism due to the higher temperature. Other possibility could resid in fact that the tested temperature was lower than “masculinizing” temperature (over 32 °C with best results around 36 °C) (Azaza et al., 2008, Baroiller and D'Cotta, 2018), therefore, not high enough to make difference in masculinization rate over the use of MT on diet, apparently.

4.5.3 MT water residues

Using a concentration of 10 mg of MT · Kg^{-1} of feed in the masculinization of Nile tilapia in BFT at 28 °C, considering the amount of feed that entered the system, it was possible to verify a reduction from 103.71 μg · L^{-1} of MT to 26.30 μg · L^{-1} of MT when comparing the same procedure being carried out using a concentration of 40 mg of MT · Kg^{-1} of feed. Ramírez et al (2024) using 30 mg of MT · Kg^{-1} of feed added the equivalent of 93 μg · L^{-1} of MT over 28 days of experiment in the masculinization of tilapia in BFT with zero water exchange. Furthermore, at 10 mg of MT · Kg^{-1} concentration and 25 °C of temperature, there would be a reduction from 26.30 μg · L^{-1} to 9.14 μg · L^{-1} of MT when compared to a 28 °C of temperature. This means a reduction in MT of almost 4 times when reducing the concentration from 40 mg MT · Kg^{-1} to 10 mg MT · Kg^{-1} at a temperature of 28 °C, and at 10 mg MT · Kg^{-1}, a reduction of almost 3 times at a temperature of 25 °C when compared to 28 °C.

For both temperatures (25 °C and 28 °C) the analysis of MT in the water, 2 hours after the last supply of feed with hormone, did not detect the presence of MT. This result indicates that temperature will not affect the degradation of MT, in the range of temperatures studied, since a natural degradation of MT probably occurs. For this study, the increased sensitivity of the HPLC technique was possible due to the use of the SPE column for extraction, purification and extraction of the samples, with the quantification and detection limits of MT being 50 μg·L^{-1} and 10 μg·L^{-1}, respectively, an improvement compared to the method used by Ramírez et al. (2024), in which the liquid-liquid technique was used and the quantification and detection limits of MT were 200 μg·L^{-1} and 50 μg·L^{-1}, respectively. This 5-fold increase in detection sensitivity was important to ensure greater safety and reliability regarding the absence of MT under the conditions presented. All possible concentrations after the introduction of feed containing MT over 28 days, either at 28 °C or 25 °C, except for the concentration of 10 mg MT · kg^{-1} at 25 °C, were higher than the detection limit used. This means that there was some degradation process that prevented the accumulation of MT in the water, and that this process was not affected by the variation in water temperature.

The absence of MT at the end of the masculinization period of Nile tilapia in the BFT system is probably due to a set of factors, which may be due to the transformation into metabolites after ingestion and elimination by the fish, or due to environmental

processes. The degradation of MT resulting from photodegradation (Shore and Shemesh, 2003; Biswas et al., 2013) is unlikely to have occurred due to the difficulty of light penetration caused by the high turbidity characteristic of BFT, just as the adsorption of MT on sediment particles (Ong et al., 2012) should also not have occurred, since the water collected for MT analysis contained materials suspended by the action of bubbles caused by aeration and due to the protocol for MT extraction analysis, which considers this possibility. Therefore, it is assumed that the elimination of MT in water occurred as a consequence of the activity of microorganisms capable of assimilating steroids (Green and Teichert-Coddington, 2000) and degrading MT (Kolok and Sellin, 2008).

Pimelobacter simplex bacteria, belonging to the genus *Nocardioidaceae*, and bacteria from the genus *Mycobacterium*, founded in aquaculture production, can convert MT into methandrostenolone (ME2) (Homklin et al., 2012; Baroiller and D'Cotta, 2018). Furthermore, *Rhodococcus equi*, *Nocardioides aromaticivorans*, and *Nocardioides nitrophenolicus* were identified as MT degraders until 10 mg/ L^{-1} (Homklin et al., 2012) and bacterial strains *Acinetobacter radioresistens* B051 and *Nocardioides nitrophenolicus* S303 have a short degradation half-life and high specific degradation rates, indicating an efficient MT-degrading (Srikwan et al., 2020). According to Srikwan et al. (2020), in an aerobic environment using the *N. nitrophenolicus* S303 strain in the MT degradation process, the following was identified: ME2 as the main intermediate metabolite in the period of 30-90 hours; the participation of the enzyme 1,2-dehydrogenase; and the absence of metabolites with androgenic activity after 264 hours.

Therefore, it is suggested that further studies be carried out to verify the feasibility of using aerobic bacterial strains as bioremediators in effluents containing MT. Thus, the inoculation of MT-degrading bacteria in water could be part of a tilapia masculinization protocol in BFT with zero water exchange, making this process more sustainable and freer of MT contamination in the environment.

The absence of MT in the water 2 hours after the end of the hormonal treatment period of Nile tilapia in the BFT system reinforces the possibility of the absence of environmental impacts caused by MT in masculinization, contrasting with what may occur when this practice is carried out in fish ponds. Although most likely all MT that entered the system was either biotransformed after being ingested by the fish or degraded

by bacterial action, further studies are still important to verify whether there is no presence of MT at levels below the detection limit of 10 μg L^{-1} after masculinization of tilapia in BFT.

In order to better understand the processes under which MT is being eliminated, it would be interesting to conduct studies to identify and quantify the metabolites that are formed and to confirm the participation of bacterial activity in the degradation of MT during the masculinization of tilapia in BFT. With this, we come ever closer to producing an environmentally responsible masculinization model, since the use of MT is still widely used in tilapia production around the world, and, in most cases, it is carried out in ponds, which generate effluents that may contain MT residues and cause environmental impacts. In a scenario of unstable international trade that may occur, developing a clean technology that overcomes possible trade barriers becomes increasingly necessary and important.

4.6 Conclusion

Results show that the intra-laboratory validation of the analytical method for detection and quantification of methyltestosterone by high performance liquid chromatography using 245 nm UV absorbance have had an excellent applicability for the residues analysis in biofloc water.

This study demonstrated that it is possible to perform the masculinization procedure of Nile tilapia in the BFT system with zero water exchange, reducing the concentration of MT most used in production, 60 mg · Kg^{-1} of feed, to one sixth, 10 mg · Kg^{-1} of feed, at 25 ˚C or 28 ˚C. However, using 25 °C the use of MT is lower, that is, it reduces the possibility of environmental impacts. We also concluded that 2 hours after the last meal containing hormone there is no presence of MT residue in the water, which shows that the system itself, probably through bacteria in biofloc, is capable of performing this function, even at 25 °C.

4.7 Acknowledgements

This research received financial support from FAPEMIG, CAPES and CNPq (National Council for Scientific and Technological Development).

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5. CONSIDERAÇÕES FINAIS

São recentes os trabalhos que tentam verificar a possibilidade de redução da concentração da MT em sistemas fechados e, com isso, reduzir os impactos ambientais. Essas respostas são importantes para o setor de produção de tilápia devido a exigências internacionais e pressões dos consumidores por produtos cuja produção está em conformidade com a sustentabilidade.

Visando uma maior sustentabilidade para o processo de masculinização, Ramírez et al. (2024) realizaram a masculinização de larvas de tilápia do Nilo em bioflocos com troca zero de água, sistema que pode ser ainda mais sustentável que o RAS. Testando as concentrações 60, 50, 40 e 30 mg MT. Kg^{-1} de ração, os autores chegaram à conclusão que utilizando 30 mg MT. Kg^{-1} de ração, valor equivalente à metade da concentração normalmente usada em viveiros (60 mg MT. Kg^{-1} de ração), é possível alcançar uma taxa de masculinização superior a 99% (Capítulo II).

Devido à concentração de 30 mg MT. Kg^{-1} de ração ter sido suficiente para uma masculinização eficaz, com 99% de masculinização (Ramírez et al., 2024), então, foi realizado um segundo experimento (Capítulo III), reduzindo ainda mais a concentração de MT na água, intencionando obter uma sustentabilidade ainda maior. Além disso, um fato em especial chamou a atenção em Ramirez et al. (2024), e deu origem a um novo

questionamento, que era tentar entender o motivo pelo qual o tratamento controle, cujas pós-larvas receberam ração isenta de hormônio, apresentou taxa de machos (74%) notadamente superior ao esperado, que corresponderia à metade dos animais. Assim, foi formulada a hipótese de que a temperatura média de 28°C, mas com picos de 31°C em dias mais quentes, no experimento de Ramírez et al. (2024), realizado em BFT com troca zero de água, pudesse ter contribuído com essa taxa elevada de machos, inclusive interferindo no tratamento controle. Com o intuito de elucidar essas questões no segundo experimento, apresentado no capítulo III, foram testadas concentrações de MT na ração ainda menores e a temperatura foi incluída como um fator a ser estudado.

Desta forma, foram avaliadas as concentrações 10, 20, 30 e 40 mg MT. Kg^{-1} de ração combinadas com as temperaturas 25°C e 28°C. À temperatura de 25°C as taxas de masculinização não diferiram entre si nas diferentes concentrações de MT, e a menor taxa de masculinização encontrada foi de 98,7%. Também não houve diferença entre as taxas de masculinização a 25°C e às taxas obtidas nas diferentes concentrações de MT a 28°C. Entretanto, à temperatura 28°C houve uma menor taxa de masculinização para as concentrações abaixo de 30 mg MT. Kg^{-1} de ração, ainda assim, produzindo taxas de 96,9 % e 97,0% para as concentrações 20 e 10 mg MT. Kg^{-1} de ração, respectivamente, que podem ser consideradas elevadas e satisfatórias para o setor de produção.

Uma das conclusões mais importantes deste experimento é que no ambiente com água a 25°C há uma redução do metabolismo dos peixes e, com isso, o consumo é reduzido e o crescimento dos peixes ocorre mais lentamente, sendo necessário uma quantidade muito inferior de ração. Com a redução do uso de ração, que é calculada em função do peso vivo dos animais, houve também uma menor entrada de MT na água, resultando em uma redução dos impactos ambientais. Contudo, ao final de 28 dias, apesar do menor impacto ambiental com menos uso de hormônio à 25°C, nesta temperatura os peixes atingem peso (277,38 mg) e tamanho (2,34 cm) inferiores ao peso (752,78 mg) e tamanho (3,33 cm) dos que foram masculinizados à 28°C. Ou seja, os peixes mantidos à temperatura da água de 25°C atingem um tamanho 2,7 vezes menor em relação a peixes que estavam na temperatura a 28°C, podendo resultar na necessidade de maior tempo e maiores custos até que os animais atinjam o peso adequado para venda.

O setor aquícola tem se esforçado cada vez mais para adequar as produções às exigências e tendências do mercado consumidor. Os produtores tendem a aderir às tecnologias que são mais viáveis do ponto de vista econômico. Contudo, as pesquisas devem atender não somente às demandas dos produtores, mas também reconhecer as tendências dos consumidores. Com isso, abandonar a masculinização realizada em viveiros e migrar para um sistema como o RAS ou o BFT é um avanço para o setor, principalmente devido a possibilidade de reduzir o impacto ambiental que o resíduo hormonal pode causar. A redução das concentrações de metiltestosterona usadas na dieta no BFT, representa um ganho ainda maior para o produtor com a possibilidade de utilizar a concentração de 10 mg $MT.Kg^{-1}$ de ração à 28°C. O uso de menor quantidade de hormônio na ração pode ser combinado com o uso de temperaturas mais baixas que 28°C e até 25°C, fazendo com que o metabolismo seja mais lento, acarretando no menor consumo de ração, o que, por consequência, impacta na menor entrada de hormônio na água, trazendo mais sustentabilidade ao processo de masculinização. Embora mais sustentável ambientalmente, o uso de temperaturas mais baixas neste manejo acarreta em peixes menores ao final, podendo gerar um maior custo ao produtor, o que pode ser averiguado, futuramente, em experimentos com análise de viabilidade econômica. Concluímos ainda, que as técnicas de HPL aqui validadas foram inéditas, inovadoras e adequadas para a detecção de MT em amostras coletadas após a masculinização em BFT. Os resultados obtidos nesta tese indicam a possibilidade de diminuição ou eliminação, por completo, de qualquer tipo de impacto para o meio ambiente utilizando o BFT com troca zero para a masculinização de tilápia do Nilo devido à ausência de MT na água após 2 horas da última alimentação com MT. Essa ausência de MT abre caminhos para se entender os processos de biotransformação e formação de metabólitos após a ingestão e de degradação causada por bactérias ambientais, que podem ser, futuramente, utilizadas intencionalmente neste tipo de sistema.