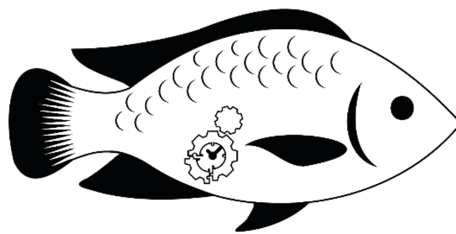
UNIVERSIDADE FEDERAL DE MINAS GERAIS
Escola de Veterinária
Programa de Pós-graduação em Zootecnia
Área de concentração Nutrição Animal/Aquacultura

Amanda Hastenreiter do Espírito Santo

RITMOS DIÁRIOS DO METABOLISMO E FISIOLOGIA DIGESTIVA DE LARVAS DE TILÁPIA (*Oreochromis niloticus*) EM DIFERENTES TEMPERATURAS



Belo Horizonte
2020

Amanda Hastenreiter do Espírito Santo

RITMOS DIÁRIOS DO METABOLISMO E FISIOLOGIA DIGESTIVA DE LARVAS DE TILÁPIA (*Oreochromis niloticus*) EM DIFERENTES TEMPERATURAS

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 Doutora em Zootecnia Área de concentração: Nutrição Animal/Aquacultura

Orientadora: Dra. Paula Adriane Perez Ribeiro

Belo Horizonte

2020

Espirito Santo, Amanda Hastenreiter do, 1991 -
E77r Ritmos diários do metabolismo e fisiologia digestiva de larvas de Tilápia (*Oreochromis niloticus*) em diferentes temperaturas / Amanda Hastenreiter do Espirito Santo . – 2020.
114f.: il.

Orientadora: Paula Adriane Perez Ribeiro
Tese (Doutorado) apresentada à Escola de Veterinária da Universidade Federal de Minas Gerais para obtenção do título de Doutora em Zootecnia.
Área de concentração: Nutrição animal/ Aquacultura.
Bibliografia: f. 36 a 43; f. 68 a 73; f. 96 a 102.
Anexos: f. 104 a 114.

1. Tilápia – Peixe - Teses - 2. Alimentação e rações - Teses - 3. Dieta em veterinária – Teses 4. Peixe – Larva –Teses - I. Ribeiro, Paula Adriane Perez - II. Universidade Federal de Minas Gerais, Escola de Veterinária – III. Título.

CDD – 636.085

Bibliotecária responsável Cristiane Patrícia Gomes – CRB2569
Biblioteca da Escola de Veterinária, Universidade Federal de Minas Gerais

ESCOLA DE VETERINÁRIA DA UFMG
COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA
Av. Antônio Carlos 6627 - CP 567 - CEP 30123-970 - Belo Horizonte- MG
TELEFONE (31)-3409-2173

www.vet.ufmg.br/academicos/pos-graduacao
E-mail cpgzootec@vet.ufmg.br

ATA DE DEFESA DE TESE DE AMANDA HASTENREITER DO ESPIRITO SANTO

Às 08:30 horas do dia 19 de fevereiro de 2020, reuniu-se, na Escola de Veterinária da UFMG a Comissão Examinadora de Tese, indicada pelo Colegiado em reunião no dia 05/12/2019, para julgar, em exame final, a defesa da tese intitulada: Ritmos diários do metabolismo e fisiologia digestiva de larvas de tilápia (Oreochromis niloticus) em submetido a temperatura constante, como requisito final para a obtenção do Grau de **Doutor em Zootecnia área de concentração Nutrição Animal – Aquacultura.**

Abrindo a sessão, a Presidente da Comissão, Prof.ª. Paula Adriane Perez Ribeiro, 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:

	Aprovada	Reprovada
Prof.(a)/Dr.(a) Felipe Guedes de Araújo	X	
Prof.(a)/Dr.(a) Leandro Santos Costa	X	
Prof.(a)/Dr.(a) Cinthia Nakayama	X	
Prof.(a)/Dr.(a) Galileu Crovatto Veras	X	
Prof.(a)/Dr.(a) Paula A. Perez Ribeiro	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 10 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, 19 de fevereiro de 2020.

Assinatura dos membros da banca:

(Vide Normas Regulamentares da defesa de Tese no verso)
(Este documento não terá validade sem assinatura e carimbo do Coordenador)
Doutorado/Atadefesa.doc

A minha avó, Maria Raimunda da Conceição, que não pôde me acompanhar até o fim dessa jornada, mas que continua me incentivando e apoiando na construção da minha carreira acadêmica.

Agradeço

A Deus, por me dar sabedoria e colocar pessoas iluminadas em meu caminho.

Aos meus pais e irmão por aceitarem e apoiarem todas as minhas decisões durante minha formação profissional.

A todos os meus familiares, que acreditaram em mim, em especial a minha prima Dayane que sempre se colocava a disposição.

À professora Dra. Paula Adriane Perez Ribeiro pela orientação, confiança, paciência, amizade durante todos esses anos e por me fazer manter a calma quando necessário.

Ao coorientador Dr. Ronald Kennedy Luz pela atenção dedicada durante o período de doutorado.

Ao Dr. Leandro Costa Santos, pela amizade e apoio.

À equipe de técnicos, professores e alunos do LAQUA.

À equipe de nutrição pelo apoio e colaboração.

Aos amigos e companheiros de caminhada Angélica, Camila, Deliane, João Paulo, Luanna, Raphael, Walisson, por todos os momentos de boas conversas e risadas. Temos muita sorte de poder passar por essa etapa juntos e nos apoiarmos!

À Universidade de Murcia, que abriu suas portas para mim.

A todos do departamento de fisiologia animal da Universidade de Murcia, que me acolheram tão bem, me fazendo esquecer, por alguns instantes, que estava a 7mil km de distância.

A Gonzalo e Oli, pela disponibilidade, ensinamentos e ajuda durante todo o processo experimental e análises.

A Yane pela ajuda durante o experimento e por ser minha companhia durante a coletas noturnas.

A Juan Fernando, que desde o primeiro momento se dispôs a me ajudar se tornando meu grande amigo e anjo de Murcia.

A Piky e Raquel, por todas as conversas, abraços e palavras de ânimo e conforto.

Ao Dr. José Fernando López-Olmeda, pela confiança, coorientação, paciência e todos os ensinamentos necessários para a construção deste trabalho.

Ao Dr. Javier pela oportunidade e apoio oferecido durante meu período em Murcia.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro e concessão da bolsa no período Sandwich.

A todos que de alguma maneira contribuíram para a realização deste trabalho.

À Instituição, pelo incentivo à pesquisa.

Muito obrigada!

“Nós todos temos sonhos. Mas, para tornar os sonhos realidade, é preciso uma enorme quantidade de determinação, dedicação, autodisciplina e esforço.” Jesse Owens

“Agora estou prestes a ir pelo caminho de toda a terra. Vocês sabem, lá no fundo do coração e da alma, que nenhuma das boas promessas que o Senhor, o seu Deus, fez deixou de cumprir-se. Todas se cumpriram; nenhuma delas falhou.” JS 23:14

RESUMO

Para apresentar um bom desempenho, as larvas devem conseguir assimilar os nutrientes da dieta, e para isso é necessário, entre alguns fatores, a presença de enzimas digestivas e metabólicas. A temperatura da água, além de ser um dos principais sincronizadores do ritmo circadiano, também é um dos fatores ambientais que mais influenciam no desenvolvimento embrionário e larvário dos peixes. Ela pode influenciar na expressão enzimática de enzimas digestivas e metabólicas, secreção de hormônios, desempenho e sobrevivência dos animais. Os fatores ambientais apresentam variações cíclicas ao largo do dia. Entre essas variações, as altas temperaturas aparecem durante o dia e as baixas temperaturas durante a noite, formando assim um termociclo. Sendo assim, o objetivo desse estudo foi avaliar a influência de diferentes temperaturas (termociclo e constante) no comportamento fisiológico, e crescimento de larvas de tilápia do Nilo (*Oreochromis nilóticos*). Para isso, ovos de tilápia foram fecundados e colocados imediatamente em incubadoras em dois sistemas distintos. Um sistema com temperatura constante (CTE) de 28ºC e um com termociclo (TC) de 31ºC: 25ºC. Ambos os sistemas ligados em um sistema de recirculação de água com filtro mecânico e biológico. O fotoperíodo foi de 12:12 LD (claro/escuro), com luzes acesas às 9:00h (Zeitgeber 0). As larvas eram alimentadas em horários fixos, quatro vezes ao dia, como uma dieta semi purificada contendo 42% de proteína bruta e 4,100 Kcal/kg de energia bruta. Larvas com 4, 8 e 13 dias pós fertilização (dpf) foram coletadas em ambos os tratamentos, em um intervalo de 4 horas ao longo de um ciclo de 24 hora para posteriores análises. O nível de expressão genica relativa das enzimas, foi analisada por qPCR. O crescimento das larvas foi avaliado através do seu comprimento padrão (experimento 1). Experimento 1: As larvas mantidas em TC apresentam um maior crescimento (teste-t, $p<0.05$) quando comparadas com as larvas mantidas em CTE. Os ritmos diários (Cosinor, $p <0,05$) da expressão dos genes *quimiotripsina, lipase, maltase, isomaltase, colecistoquinina* (*cck*) *e neuropeptídeo y (npy*) foram demostrados para larvas com 13dpf mantidas em TC, com picos de expressões (acrofase $p <0,05$) próximos as fases de alimentação. Experimento 2: As larvas com 13 dpf, mantidas em um TC apresentam diferenças significativas (Cosinor, $p <0,05$), nos ritmos diários na expressão relativa do gene *alanina aminotransferase* (*alt*), *aspartato aminotransferase* (ast), *enzima málica*, *glicose-6-fosfato deseidrogenase* (*g6pd*), *piruvato quinase* (*pk*) e f*osfofrutoquinase* (*pfk*). Enquanto as larvas mantidas em CTE, com a mesma idade, não apresentam ritmos diários (Cosinor, $p >0,05$). Além disso, a expressão enzimática

($p<0,05$) das larvas mantidas em TC, apresentaram acrofase entre o período de alimentação. Podemos concluir que o termociclo melhora a maturação do sistema circadiano das larvas, o que melhora sua eficiência digestiva e metabólica. Estes resultados podem ser úteis para melhorar os protocolos usados na larvicultura de tilápias aumentando o desempenho da produção.

Palavras-Chave: Expressão genica. Fisiologia metabólica. Peixes. Nutrição. Larvicultura.

ABSTRACT

Larvae have to assimilate the nutrients from the diet to get adequate growth rates and, for that purpose, it is necessary, among other factors, the presence of digestive and metabolic enzymes. Water temperature is one of the main synchronizers of biological rhythms and one of the most influential environmental factors for fish embryonic and larval development. Water temperature can influence the expression of digestive and metabolic enzymes, hormone secretion, growth and survival of animals. The environmental factors display cyclic variations along the day. Among them, high water temperature occurs during the day and low temperatures during the night, generating the thermocycle. Therefore, the objective of the present research was to evaluate the influence of different temperature regimes (thermocycle vs. constant) on the physiology and growth of Nile tilapia (*Oreochromis niloticus*) larvae. For that purpose, tilapia eggs were fertilized and immediately placed in incubators in two different rearing systems: one system with constant temperature (CTE) and one system with a thermocycle of 31ºC: 25ºC. Both were water recirculation systems equipped with mechanical and biological filters. Photoperiod was set at 12:12 LD (light/dark), with lights on at 9:00h (*Zeitgeber* time 0 h). Larvae were fed at fixed times, four times a day, with a semipurified diet of 42% of crude protein and 4,100 Kcal/kg of gross energy. Larvae at 4, 8 and 13 days postfertilization (dpf) were collected from both temperature treatments every 4 h along a 24 h cycle for further analyses. The levels of relative expression of all genes were analyzed by qPCR. Larval growth was measured through their standard length (experiment 1). Experiment 1: Larvae maintained at TC showed higher growth (t-test, $p <0.05$) than larvae kept at CTE. Daily rhythms (Cosinor, $p <0.05$) in the expression of chymotrypsin, *lipase, maltase, isomaltase, cholecystokinin* (*cck*) and *neuropeptide Y (npy*) were shown in 13dpf larvae reared in TC, with expression peaks close to the feeding phase. Experiment 2: larvae at 13 dpf and maintained at TC showed significant rhythms (Cosinor, $p <0.05$) of relative expression of the genes *alanine aminotransferase* (*alt*), *aspartate aminotransferase* (ast), *malic enzyme*, *glucose-6-phosphate dehydrogenase* (*g6pd*), *pyruvate kinase* (*pk*) and *phosphofructokinase* (*pfk*). On the other hand, larvae reared at CTE at the same stage did not present daily rhythms (Cosinor, $p >0.05$). The acrophase of expression of the enzymes in larvae maintained at TC appeared around mealtime. Therefore, it can be concluded that, under a thermocycle, tilapia larvae improve the maturation of their circadian system, which would thus improve their digestive

and metabolic efficiency. These results can be helpful for improving protocols used in tilapia temperature and increasing production performance.

Keywords: Gene expression. Metabolic physiology. Fish. Nutrition. larviculture.

LISTA DE FIGURAS

Figura 1. Produção aquícola mundial de 1990-2016… 25

Figura 2. Estrutura cerebral de um peixe… 31

CAPÍTULO 1: Effects of temperature regime on growth and daily rhythms o digestive factors in Nile tilapia (*Oreochromis niloticus*) larvae

Figure 1. Standard length (mm) of tilapia larvae on 4, 8 and 13 days post fertilization (dpf) at a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (black bars) or constant temperature (CTE) of 28 °C (white bars). Different upper case and lower case letters indicate statistically significant differences between developmental stages (dpf) within the TC or CTE groups, respectively (two-way ANOVA)…………………………………………………………………………..…55

Figure 2. Daily variations in the relative mRNA expression (fold change) of pepsinogen analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by White squares, □). Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times……….………………….……58

Figure 3. Daily variations in the relative mRNA expression (fold change) of chymotrypsinogen analyzed in tilapia larvae on 4 (A) and 13 (B) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by White squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate

statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times…………………………………….…58

Figure 4. Daily variations in the relative mRNA expression (fold change) of trypsinogen analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by White squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times..………………………………………………………………………………59

Figure 5. Daily variations in the relative mRNA expression (fold change) of lipase analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. ………..…..…...59

Figure 6. Daily variations in the relative expression of *maltase* expression analyzed in tilapia larvae at 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) under a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by the black circles, ●) or constant temperature (CTE) of 28ºC (represented by the white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever a Cosinor test was significant (p<0.05). No significant differences were observed between groups and time points (two-way ANOVA, p>0.05). The white and black bars above each graph represent the light phase and dark phase, respectively. ……………60

Figure 7. Daily variations in the relative expression of *isomaltase* expression analyzed in tilapia larvae at 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) under a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by the black circles, ●) or constant temperature (CTE) of 28ºC (represented by the white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever a Cosinor test was significant (p<0.05). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, p<0.05). The white and black bars above each graph represent the light phase and dark phase, respectively……………………………………………………………………60

Figure 8. Daily variations in the relative expression of *neuropeptide Y* (*npy*) expression analyzed in tilapia larvae at 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) under a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by the black circles, ●) or constant temperature (CTE) of 28ºC (represented by the white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever a Cosinor test was significant (p<0.05). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, p<0.05). The white and black bars above each graph represent the light phase and dark phase, respectively. …………………………………………………61

Figure 9. Daily variations in the relative expression of *cholecystokinin* (*cck*) expression analyzed in tilapia larvae at 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) under a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by the black circles, ●) or constant temperature (CTE) of 28ºC (represented by the white squares, □). The sinusoidal dashed line represents the adjustment

to a sinusoidal rhythm whenever a Cosinor test was significant (p<0.05). No significant differences were observed between groups and time points (two-way ANOVA, p>0.05). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onse……………………………………………………………………………………61

Figure 10. Map of the acrophases of the digestive factors and hormones analyzed in tilapia larvae from 4, 8 and 13 days post fertilization (dpf), under a 12:12LD cycle and two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC or constant temperature (CTE) of 28ºC. The acrophases from larvae reared in the TC group at 4, 8 and 13 dpf are represented by the black squares, triangles and circles, respectively. The acrophases from larvae reared in the CTE group at 4, 8 and 13 dpf are represented by the white squares, triangles and circles, respectively. The acrophase is indicated only for the statistically significant rhythms (Cosinor p<0.05). The name of each represented gene is indicated to the left. The white and black bars above the graph represent the light and dark phases, respectively. Time scale(x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset…………………………………………………………………………..………...62

CAPÍTULO 2: Ontogenia dos ritmos diários de fatores metabólicos da tilápia do Nilo (*Oreochromis niloticus*), submetida a temperatura constante e termociclo

Figura 1. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de alanina aminotransferase (alt) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de

alimentação..………………………………………………………………………………87

Figura 2. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de aspartato aminotransferase (ast) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação……………………………………………………………......87

Figura 3. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de enzima málica em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação…... 88

Figura 4. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de glicose-6-fosfato desidrogenase (g6pd) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de

alimentação………………………………………………………..……………………88

Figura 5. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de fosfofrutoqinase (pfk) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação……………………………………………………………...….....89

Figura 6. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de piruvato quinase (pk) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação……………………………………………………………………...89

Figura 7. Mapa das acrofases dos fatores digestivos e hormônios analisados em larvas de tilápia de 4, 8 e 13 dias pós-fertilização (dpf) em um ciclo LD 12:12 e por dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C ou temperatura constante (CTE) de 28 ° C. As acrofases das larvas criadas no grupo TC em 4, 8 e 13 dpf são representadas por quadrados pretos, triângulos e círculos, respectivamente. As acrofases das larvas criadas no grupo CTE em 4, 8 e 13 dpf são representadas por quadrados brancos, triângulos e círculos, respectivamente. A acrofase é indicada apenas para os ritmos estatisticamente significativos (Cosinor, p <0,05). O nome de cada gene representado é

indicado à esquerda. As barras brancas e pretas acima do gráfico representam as fases de claro e escuro, respectivamente. As setas acima do gráfico indicam os tempos de alimentação para grupos de 8 e 13 dpf. A escala de tempo (eixo x) é expressa como Tempo Zeitgeber (ZT), em que ZT 0 h corresponde ao início da luz……………………………………….…90

LISTA DE TABELAS

Tabela 1. Principais espécies produzidas na aquacultura mundial…………………....…...26

CAPÍTULO 1: Effects of temperature regime on growth and daily rhythms of digestive factors in Nile tilapia (*Oreochromis niloticus*) larvae

Table 1. Primer sequences used for the quantitative analysis by qPCR.………………….56

Table 2. Acrophase and statistical significance values of the genes subjected to Cosinor analysis.…………………………………………………...……………..………...57

CAPÍTULO 2: Ontogenia dos ritmos diários de fatores metabólicos da tilápia do Nilo (*Oreochromis niloticus*), submetida a temperatura constante e termociclo

Tabela 1. Formulação da dieta experimental…….……………………………..…….…...85

Tabela 2. Sequência de primers utilizada para análise de qPCR quantitativo…….……..86

Tabela 3. Acrofase dos genes relacionados ao metabolismo de tilápia do Nilo, frente a um termocilo (31ºC: 25ºC) e uma temperatura constante (28 ºC) ……………………....…86

LISTA DE SIGLAS

AAA-Atividade antecipatória de alimento

ADP-Adenosina difosfato

ALT-Alanina aminotransferase

ANOVA- Análise de variância

ATP- Adenosina Trifosfato

AST- Aspartato aminotransferase

CAPES- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCK – Colecistoquinina

cDNA- Ácido desoxirribonucleico complementar

CNPq- Conselho Nacional de Desenvolvimento Científico e Tecnológico

CTE- Constante

DNA- Ácido desoxirribonucleico

Dpf- Dias pós fertilização

FAO- Food and agriculture organization

Fw- Foward

G6PD- Glicose 6 fosfato desidrogenase

HCG- Gonadotrofina coriónica humana

mRNA- Ácido ribonucleico mensageiro

NADPH- Fosfato de dinucleotídeo de adenina e nicotinamida

NPY – Neuropéptido Y

NSQ- Núcleo supraquiasmático

PCR – Polymerase chain reaction – Reação em cadeia de polimerase

PFK- Fosfofrutoquinase

PK- Piruvato quinase

qPCR- Quantitative polymerase chain reaction- Reação em cadeia de polimerase quantitativa

RNA -Ácido ribonucleico

Rv -Reverse

TC- Termociclo

Zt- Zeitgeber

SUMÁRIO

1. **INTRODUÇAO GERAL**…………………………………………………...……….24
2. **REVISÃO DE LITERATURA**…………………………………………….……..24
   - **2.1 Tilápia (Oreochromis niloticus)** …………………………………………...…24
   - **2.2 Processos digestivos e metabólicos em peixes**………………………………26
   - **2.3 Ritmo biológico** …………………………………………………………...….29
   - **2.4 Ritmos circadianos na alimentação e digestão** ……………………………..31
   - **2.5 Influência dos ciclos de temperatura nos ritmos biológicos e desenvolvimento larvário** ……………………………………………………………………..33
3. **OBJETIVOS**……………………………………………………………………34
   - **3.1 Objetivo geral** ……………………………………………………………...34
   - **3.2 Objetivos específicos** ……………………………………………………….34
4. **REFERENCIAS BIBLIOGRÁFICAS** …………………………………………….36

CAPÍTULO 1- Effects of temperature regime on growth and daily rhythms of digestive factors in Nile tilapia (*Oreochromis niloticus*) larvae

1. **INTRODUCTION** ……………………………………………………………..46
2. **MATERIALS AND METHODS** ……………………………………………..48
   - **2.1 Animals and experimental design** …………………...…………...………..…48
   - **2.2 Larval growth** ………………………………………………………...……...50
   - **2.3 Real-time RT-PCR analysis** ……………………………….………...…………50
   - **2.4 Data analysis**…………………………………………………...…………..51
3. **RESULTS**………… …..…………………………………………….…...……..51
   - **3.1 Hatching timing and rate**……………………………………...………..51
   - **3.2 Larval growth** ………………………………..………………….……..52
   - **3.3 Pepsinogen expression** ………………………………….………………52
   - **3.4 Chymotrypsinogen expression**……………………………………………....52
   - **3.5 Trypsinogen expression**…………………………………………….…..53
   - **3.6 Lipase expression**……………………………………………….…....53
   - **3.7 Maltase expression**……………………………………….……..……53

3.8 Isomaltase expression……………………………………………………..….….……54
3.9 Npy expression………………………………………...……………………...54
3.10 Cck expression…………………………………………………………….……54
4. DISCUSSION…………………………………………………………….…………64
5. CONCLUSIONS…………………………………………………...………..67
6. ACKNOWLEDGMENTS…………………………………………..………67
7. REFERENCE…………………………………………………………….….68

CAPÍTULO 2- Ontogenia dos ritmos diários de fatores metabólicos da tilápia do Nilo (*Oreochromis niloticus*), submetida a temperatura constante e termociclo

1. INTRODUÇÃO……………………………………………………….……..78
2. MATERIAL E MÉTODOS………………………………………….….….…..79
2.1 Animais e design experimental…………………………………….…...79
2.2 Análise RT-PCR em tempo real……………………….……………..81
2.3 Análise de dados………………………………………………………82
3. RESULTADOS…………………………………………………….……...83
3.1 Expressão de alanina aminotransferase…………………………….…83
3.2 Expressão de aspartato aminotransferase……………………………..83
3.3 Expressão de enzima málica…………………………………………83
3.4 Expressão de glicose-6-fosfato desidrogenase……………………………84
3.5 Expressão de fosfofrutoquinase……………………………………...84
3.6 Expressão de piruvato quinase……………………………………85
4. DISCUSSÃO ……………………………………………………………...92
5. CONCLUSOES……………………….………………………………..95
6. AGRADECIMENTOS….………………………………………………...95
7. REFERENCIAS ……..…………………………………..……………96
8. CONSIDERAÇOES FINAIS….…………………………………………..103
ANEXOS………………………...……………………………………104

1. INTRODUÇÃO GERAL

A tilápia é a quarta espécie de peixe mais produzida mundialmente, devido aos seus excelentes índices produtivos, como ciclo de produção curto, rápido crescimento e tolerância a condições sub-ótimas de cultivo.

O crescimento dos peixes é um processo complexo e pode ser afetado por fatores ambientais, fisiológicos, comportamentais, entre outros. Um dos fatores ambientais que mais influência no crescimento destes animais é a temperatura, uma vez que eles são organismos ectotérmicos. A temperatura tem grande impacto na fisiologia animal, podendo atuar no metabolismo, absorção de nutrientes e outros fatores relacionados diretamente ao crescimento dos peixes.

A eficiência da conversão de alimentos em nutrientes pode depender da disponibilidade de enzimas envolvidas nesse processo (Jobling 1995; Perez-Casanova et al., 2006). Portanto, o conhecimento das principais enzimas envolvidas na digestão e metabolismo, bem como seus mecanismos de atuação no organismo dos peixes nos permite entender diversos aspectos de sua fisiologia.

Os animais apresentam ritmos biológicos resultantes de um longo processo evolutivo, causado por mudanças climáticas e pela rotação da terra (dia/noite). Os ritmos biológicos são controlados por sincronizadores, e no caso dos peixes, os mais evidenciados na literatura são luz, temperatura e alimentação.

O conhecimento sobre a fisiologia digestiva e metabólica dos peixes e os fatores que podem influenciá-la, nos permite ajustar os protocolos de produção, adequando-os para cada estágio de crescimento, resultando assim, em maiores índices produtivos e maior rentabilidade da atividade.

2. REVISÃO DE LITERATURA

2.1 Tilápia *(Oreochromis niloticus)*

Segundo a FAO (2018), a produção mundial aquícola em 2016, incluindo as plantas aquáticas, chegou a 110,2 milhões de toneladas, atingindo um valor de venda de 243.500 milhões de dólares. Dentro desse montante, a produção de peixes foi de 54,1 milhões de toneladas (138.500 milhões de dólares) (Figura 1). De 2001 a 2016 foi observado um

crescimento anual na aquicultura mundial de 5,8%.

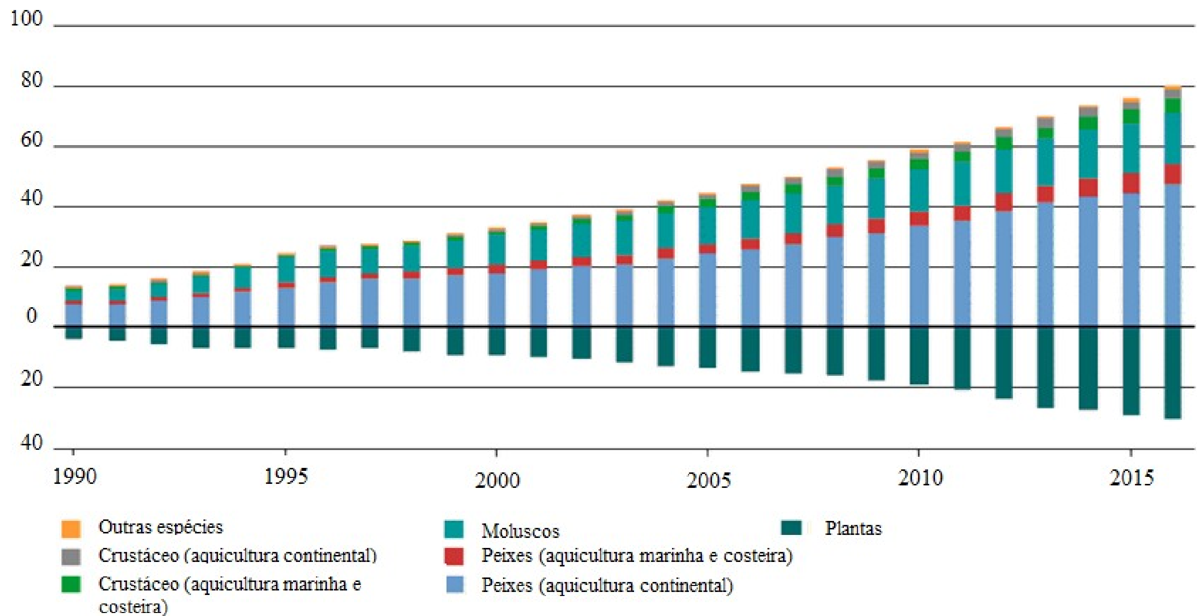


Figura 1. Produção aquícola mundial de 1990-2016 (Adaptado de FAO, 2018).

A tilápia *(Oreochromis niloticus)* e outros ciclídeos apresentaram um aumento constante na produção mundial, alcançando 1,6 milhões de toneladas em 2016, duplicando os valores registrados no ano de 2005. A tilápia aparece como a quarta espécie mais produzida mundialmente (Tabela 1), sendo as três primeiras posições ocupadas pelas carpas, que apesar de serem mais cultivadas, têm a produção considerada estável (FAO, 2018).

No Brasil, a tilápia foi introduzida pela primeira vez em 1971, pelo DNOCS – Departamento Nacional de Obras Contra a Seca; porém. Apenas na década de 1990, com a difusão das técnicas de cultivo, a produção começou a se estruturar (Nogueira e Rodrigues, 2007).

A tilápia apresenta excelentes índices produtivos (Schwarz et al., 2011), como ciclo de produção curto, rápido crescimento (Nandlal e Pickering, 2004), tolerância a condições sub-ótimas de cultivo, como variações no pH, temperatura e oxigênio, e possui, ainda, excelente carne e ótima aceitação no mercado. Além disso, é uma espécie que aceita uma grande variedade de alimentos (Nogueira e Rodrigues, 2007; Nandlal e Pickering, 2004), respondendo com eficiência à ingestão de proteínas de origem vegetal e animal, além de serem resistentes a doenças, superpovoamentos e desovarem durante todo ano (Nogueira e Rodrigues, 2007).

A tilápia se destaca das demais espécies por adequar-se à indústria de filetagem, devido à ausência de espinhos intramusculares em “Y” (Meurer et al., 2003), possuir rendimento de filé de aproximadamente 33%, apresentar carne de textura firme, branca e de elevado valor

nutricional.

Tabela 1. Principais espécies de peixes produzidas na aquacultura mundial.

Espécies	2010	2012	2014	2016
Carpa herbívora *Ctenopharyngodon idellus*	4.362	5.018	5.539	6.068
Carpa prateada *Hypophthalmichthys molitrix*	4.100	4.193	4.968	5.301
Carpa comum *Cyprinus carpio*	3.421	3.753	4.161	4.557
Tilápia do Nilo *Oreochromis niloticus*	2.537	3.260	3.677	4.200
Carpa *Hypophthalmichthys nobilis*	2.587	2.901	3.255	3.527

Adaptado de FAO (2018).

2.2 Processos digestivos e metabólicos em peixes

O crescimento dos peixes é um processo complexo, afetado por fatores comportamentais, fisiológicos, nutricionais e ambientais (Martinez et al., 1996). Por serem ectotérmicos, os peixes encontram uma grande variedade de habitats e são expostos a mudanças frequentes nas condições ambientais (Dutta et al., 1994).

A temperatura ambiental tem um impacto direto sobre os aspectos fisiológicos (Koumoundouros et al., 2002) e bioquímicos, podendo aumentar o metabolismo dos peixes (Blier et al., 1997). Com a diminuição da temperatura, diminui-se também o consumo de alimento, taxa digestiva, absorção de nutrientes, excreção de nitrogênio, síntese de proteínas e taxa de crescimento (Fauconneau, 1985).

Em ambientes totalmente controlados o crescimento dos animais é limitado pela capacidade do organismo em digerir e transportar os nutrientes (Blier et al., 1997). Uma nutrição adequada durante o período larval dos peixes é fundamental para o sucesso das fases subsequentes (Hayashi et al., 2002). Sendo assim, o conhecimento do comportamento alimentar dos animais, assim como sua fisiologia digestiva, contribuirá para a otimização de dietas e protocolos alimentares, melhorando as taxas de crescimento e sobrevivência.

A eficiência da conversão de alimentos em nutrientes pode depender da disponibilidade de enzimas envolvidas nesse processo (Jobling 1998 e Perez-Casanova et al., 2006). Estudos sobre secreções digestivas em peixes podem elucidar alguns aspectos fisiológicos e contribuir para solucionar problemas nutricionais, como por exemplo, a correspondência de uma dieta

artificial que supra as necessidades nutricionais do peixe (Furné et al., 2005).

Em seguida comento sobre algumas das enzimas envolvidas na digestão e metabolismo de proteínas, lipídios e carboidratos.

Proteínas

A pepsina é a principal protease ácida presente no estômago (Malik et al., 2005), facilitando a digestão por hidrólise e desnaturação das proteínas (Lauff e Hofer, 1984), rompendo as cadeias polipeptídicas no lado aminoterminal dos resíduos de aminoácidos cíclicos (tirosina, fenilalanina e triptofano), para transformá-las em peptídeos menores (Baldisserotto et al., 2014). A pepsina é responsável pela hidrólise inicial e parcial das proteínas, juntamente com o ácido clorídrico (Baldisserotto, 2002, Baldisserotto et al., 2014). A digestão tem continuidade no intestino, com auxílio da quimiotripsina e tripsina, principais enzimas secretadas na porção anterior do intestino (Bougatef, 2013, Baldisserotto et al., 2014), sendo responsáveis por continuarem a redução dos peptídeos (Lehninger e Cox, 2006).

A tripsina é responsável pela hidrólise das ligações envolvendo os grupos carbonila da arginina e da lisina (Bougatef, 2013). É considerada uma enzima chave no processo de digestão proteica, pois ativa sua própria forma precursora juntamente com outras proteases, incluindo a quimiotripsina (Baldisserotto et al., 2014).

A quimiotripsina hidrolisa ligações peptídicas cujo grupos carbonila são oferecidos por resíduos de fenilalanina, tirosina e triptofano. Este estágio de digestão proteica é realizado com muita eficiência, uma vez que cada enzima possui diferentes especificidades por aminoácidos (Lehninger, e Cox, 2006).

Outras peptidases, continuam a hidrólise dos peptídeos no intestino. Entre elas a carboxipeptidase, que remove os resíduos carboxiterminais dos peptídeos, transformando-os em aminoácidos livres, para que possam ser transportados através das células epiteliais, pelo sangue até o fígado (Champe et al., 2006). No fígado, os aminoácidos podem ser transaminados pela alanina aminotransferase e aspartato aminatrasferase, formando L-glutamato e um α-cetoácido correspondente (Champe et al., 2006).

Lipídios

As lipases, embora possam ser encontradas no estômago de alguns peixes, apresentam seu sítio primário de hidrólise na porção anterior do intestino e cecos pilóricos (quando existentes), continuando em menor proporção em outros sítios intestinais (Baldisserotto et al.,

2014).

Os lipídios da dieta são emulsificados pela ação dos sais biliares, permitindo, assim, sua dispersão no meio aquoso. A lipase hidrolisa os triacilgliceróis em glicerol e ácidos graxos. Outras enzimas estão envolvidas neste processo, permitindo a estabilização da lipase e hidrólise dos resíduos de ácidos graxos (Motta, 2003).

Para a síntese dos ácidos graxos, que ocorre principalmente no fígado, é necessário a incorporação de carbonos, a partir de moléculas de acetil-CoA, na cadeia do ácido graxo, utilizando ATP e NADPH reduzido (Champe et al., 2006). A via das pentoses-fosfato é a maior fornecedora de NADPH para a síntese de ácidos graxos, sendo dois NADPHs produzidos para cada molécula de glicose metabolizada nesta via. O NADPH pode ser produzido por meio da conversão citossólica do malato em piruvato, onde o malato é oxidado e descarboxilado pela ação da enzima málica, uma das enzimas envolvidas na síntese de ácidos graxos (Champe et al., 2006). Outra forma da obtenção do NADPH é por meio da oxidação da glicose-6-fosfato (G6P) em 6-fosfogulono-δ-lactona, reação catalisada pela glicose-6-fosfato-desidrogenase (G6PD), redução de NADP+ a NADPH (Champe et al., 2006).

Carboidratos

A digestão dos carboidratos acontece em quase todo trato gastrointestinal dos peixes (Baldisserotto et al., 2014). Os carboidratos provenientes da dieta são hidrolisados pela ação da amilase, em maltose e isomaltose. Em seguida, estes compostos são hidrolisados em glicose por meio de enzimas como maltase e isomaltase, para assim serem absorvidos pelo intestino. A glicose entra nas células por difusão facilitada, independente de Na+, ou num sistema de co-transporte dependente de Na+ (Champe et al., 2006).

Na via glicolítica, a molécula de glicose passa por uma série de reações até ser convertida em piruvato. Essas reações estão divididas em dois estágios, o primeiro (fase preparatória), a glicose é fosforilada por dois ATPs e clivada, produzindo duas moléculas de gliceraldeído-3-fosfato. Uma das enzimas envolvida nesta fase é a fosfofrutoquinase (PFK), que catalisa a transferência de um grupo fosfato do ATP para a frutose-6-fosfato, liberando frutose-1,6-bifosfato (Lehninger e Cox, 2006; Motta, 2003). Já no segundo estágio, as duas moléculas de gliceraldeído-3-fosfato são oxidadas e fosforiladas, as reações envolvidas no processo rearranjam os fosfatos em compostos fosforilados de alta energia, que ao serem hidrolisados liberam energia livre para sintetizar ATP. Uma das enzimas envolvidas nesse estágio é a piruvatoquinase (PK), que catalisa a reação de transferência do grupo fosfato do

fosfoenolpiruvato para o ADP, formando piruvato e ATP (Lehninger e Cox, 2006; Motta, 2003).

2.3 Ritmo biológico

Segundo Kulczykowska et al. (2010), ritmos diários de luz e escuridão e ciclos sazonais de mudanças climáticas, causados pela rotação da terra, têm deixado marcas na evolução dos animais. Estas marcas são conhecidas como ritmos biológicos e só não têm influência em animais que vivem em cavernas e nas profundidades do oceano.

A característica básica dos ritmos biológicos é que eles persistem sob condições ambientais constantes, na ausência de qualquer efeito externo. Os ritmos, portanto, são denominados endógenos, que surgem dentro organismo e não são impostos pelo meio ambiente (Cymborowski, 2010).

A ritimicidade biológica foi observada pela primeira vez em 1729, quando o astrônomo francês Jean Jacques d'Ortous de Marian relatou que os movimentos das folhas de uma planta, conhecida como *mimosa*, permaneciam oscilando, num período de 24 horas, mesmo sem a presença de luz (Araújo e Marques, 2002; Roenneberg e Merrow, 2005). A persistência dos ritmos da planta significa que o relógio biológico é "endógeno" e mesmo sem um estímulo externo a movimentação persiste (Wulund e Reddy, 2015). Portanto, ritmos biológicos são definidos como qualquer evento endógeno, que se repete de maneira regular num organismo (Tavares-Dias e Mariano, 2015), ou seja, a capacidade dos seres vivos de expressarem seus comportamentos e controlarem sua fisiologia de forma recorrente e periódica (Araújo e Marques, 2002).

Os ritmos mais estudados estão relacionados às mudanças ambientais, aos quais os animais devem se adaptar (Morgan, 2004). A ritmicidade dos fatores ambientais impõe aos animais o desafio de antecipar esses eventos cíclicos, preparando-os para lidar com tais eventos, tornando os mecanismos antecipatórios uma característica inerente dos organismos vivos (Volpato e Trajano, 2006).

Os ritmos biológicos têm sido classificados de acordo com sua periodicidade. Os ciclos que se repetem em intervalos de até 20 horas são conhecidos como ultradianos (respiração, batimentos cardíacos, disparos de neurônios, entre outros) (Gonçalves e Marques, 2012); os infradianos se repetem em intervalos maiores que 28 horas (reprodução) (Caperl et al., 2003); e o ritmo circadiano, que se repete em intervalos entre 20 a 28 horas (Herrero et al., 2009; Schulz e Leuchtenberger, 2006). O ritmo circadiano (ciclo claro/escuro) é um dos mais

estudados, e mais bem compreendido nos seres vivos (Volpato e Trajano, 2006).

Normalmente o ritmo biológico não é exatamente o mesmo que o do ciclo do meio ambiente (Cymborowski, 2010). Os períodos dos ritmos endógenos na verdade diferem daqueles na natureza, mas sinais de sincronização exógenos ajustam ou "arrastam" o ritmo biológico, para que os organismos permaneçam em sintonia com o ambiente (Cymborowski, 2010; Vera et al., 2007). Alguns sincronizadores estudados para peixes são luz, temperatura e alimentação, e estes podem influenciar, como por exemplo, na natação, liberação de hormônios (Bromage et al., 2001), interação social (Nejdi et al., 1996), entre outros.

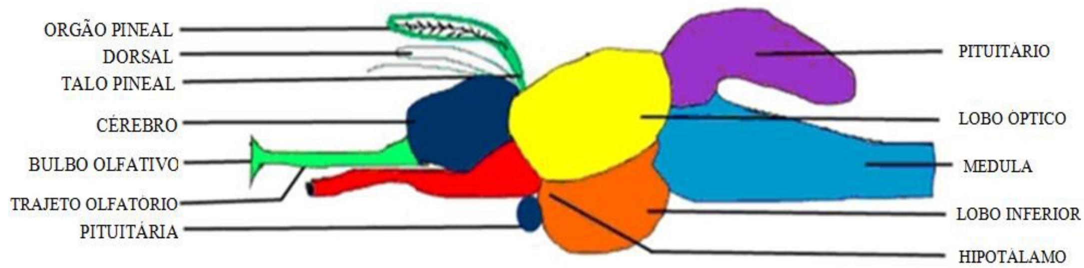
Como a maioria dos ritmos está sincronizada com os ciclos ambientais, os cronobiológicos se referem ao tempo, como definido por esses ciclos ambientais, chamando-o de Zeitgeber (ZT). Quando o animal é mantido num ciclo de 24 horas, sendo 12 horas de luz e 12 horas de escuro (LD 12:12), a zeitgeber zero ou ZT0 corresponde ao momento que a luz se acende e ZT12 corresponde ao momento que a luz se apaga. Assim, todos os pontos de tempo entre 0 e 12 se referem às horas diurnas, enquanto os pontos de 13 a 24 (o mesmo que o ponto 0), se referem a horas noturnas (Cymborowski, 2010).

Em mamíferos a sincronização ocorre por meio do núcleo supraquiasmático (NSQ) do hipotálamo, no sistema nervoso central, considerado o marcapasso central do organismo neste grupo de animais (Maitra et al., 2013). Em peixes, a existência e localização de um marcapasso central similar ao NSQ dos mamíferos ainda não foi descrito, porem alguns estudos sugerem que a glândula pineal teria um papel similar (Falcon et al., 2010).

A glândula pineal (Figura 1), está localizada abaixo do crânio, e em peixes é coberta por uma fina e pouco pigmentada camada de pele (Kulczykowska et al., 2010). Ela possui células fotorreceptoras que se assemelham aos cones da retina dos vertebrados e permitem a transmissão de informações nervosas ao centro do cérebro, liberando o hormônio melatonina no sangue (Falcon, 1999). A melatonina é uma das principais saídas dos ritmos circadianos, que transmitem informações rítmicas ao organismo. O padrão diário de secreção de melatonina é muito conservado entre os vertebrados, onde a pineal produz melatonina à noite.

A melatonina se difunde rapidamente em cada célula do organismo, interagindo com os receptores das membranas celulares (Kulczykowska et al., 2010). A síntese e a liberação de melatonina são controladas pelo ciclo claro/escuro, aumentando sua produção no escuro e diminuindo com a iluminação (Villamizar et al., 2011). Assim, o sinal de melatonina que reflete o fotoperíodo prevalecente fornece ao animal informações sobre o ciclo claro/escuro (Kulczykowska et al., 2010).

Figura 2. Estrutura cerebral de um peixe. Adaptado de Saha (2018).



O envolvimento da melatonina no controle de processos que exibem ritmos diários ou sazonais é amplamente aceito, mas ainda não está completamente compreendido (Falcon et al., 2010). Os ritmos circadianos afetados pela pineal e/ou melatonina incluem atividade locomotora, repouso, migração, pigmentação da pele, osmorregulação, metabolismo e ingestão alimentar (Falcon et al., 2007).

2.4 Ritmos circadianos na alimentação e digestão

O ciclo claro/escuro, juntamente com a alimentação e a temperatura, é um dos principais sincronizadores circadianos em peixes. Eles também são considerados cruciais para as espécies aquícolas, devido à sua influência no desempenho, fisiologia, comportamento e estresse (Kitagawa et al., 2015).

López-Olmeda e Sánchez-Vázquez (2010) fizeram a seguinte pergunta: “Por que ritmos alimentares?” O alimento dificilmente está disponível o tempo todo no ambiente natural, sendo restrito a um momento particular, período em que a disponibilidade de alimento é maior. Além disso, o animal lida com a disponibilidade de alimento e a ocorrência de predadores, que também é maior num determinado momento. Sob tal ambiente cíclico, os peixes evoluíram com mecanismos para prever o tempo de alimentação, fazendo com que seus processos fisiológicos sejam ativados antecipadamente.

Quando os animais são submetidos à alimentação programada, ou seja, um horário fixo, exibem um aumento na atividade locomotora em antecipação à próxima refeição (López-Olmeda et al., 2012[a]). Essa antecipação é conhecida como atividade antecipatória de alimento (AAA) e pode ser definida como o aumento de atividade, duas vezes ou mais, acima de uma linha de base de atividade, mantida pelo menos 30 minutos e exibida logo antes do horário de alimentação (Mistlberger, 1994).

Uma das principais características da AAA é o seu desenvolvimento gradual, com vários ciclos de alimentação sendo necessários para sua manifestação. O tempo que os peixes levam

para ressincronizar sua AAA, em função de uma mudança no tempo de alimentação, está diretamente relacionada à quantidade de horas que o horário das refeições foi deslocado (López-Olmeda e Sánchez-Vázquez, 2010). Fisiologicamente, se o organismo for capaz de antecipar uma refeição ele pode melhorar a aquisição de alimentos e nutrientes (Comperatore e Stephan, 1987; Sánchez-Vázquez e Madrid, 2001).

Vera et al. (2007) alimentaram o goldfish (*Carassius auratus*) por três semanas em um horário fixo e encontraram aumento nos níveis plasmáticos do neuropeptído Y e na atividade enzimática da amilase intestinal, duas horas antes da alimentação. Resultados semelhantes foram encontrados por Montoya et al. (2010[a]), que observaram aumento na atividade da amilase e da protease alcalina, quatro horas antes do horário da refeição da dourada (*Sparus aurata*), alimentada num horário fixo. Para o grupo alimentado em horário aleatório, os animais apresentaram aumento nas atividades enzimáticas, apenas uma hora após a refeição.

López-Olmeda et al. (2012b), ao alimentarem enguias europeias (*Anguilla anguilla*) no início da fase escura (19:00 horas), observaram aumento na atividade da protease ácida e da amilase, duas horas antes do tempo fixo de alimentação.

Prieto-Guevara et al. (2015), testaram dois horários de alimentação para o bagre híbrido (*Pseudoplatystoma reticulatum* x *Leiarius marmoratus*), 12:00 e 24:00 horas, num fotoperíodo de 12:12 L/D. A ingestão de alimentos não foi influenciada pelo horário de alimentação, porém, os autores observaram aumento na taxa de crescimento específico para o grupo de animais que recebeu alimentação diurna.

Lazado et al. (2017) observaram para o sernambiguara (*Trachinotus falcatus*) uma sincronização da alimentação com o fotoperíodo, que caracterizou uma antecipação de duas horas na atividade enzimática das proteases, leucina aminopeptidase e lipase, para os animais alimentados periodicamente às 9:00 horas, durante seis semanas. Além da atividade antecipatória, eles observaram uma maior atividade dessas mesmas enzimas, quando comparadas ao grupo de animais que recebeu alimentação aleatória.

O ritmo diário dos fatores digestivos em peixes ainda é pouco elucidado, porém, já se pode perceber a importância do estudo deste tema. Uma vez que a alimentação do animal esteja sincronizada, o organismo é capaz de se preparar para uma próxima refeição. Essa antecipação causa aumento na digestão e eficiência alimentar (Montoya et al., 2010b), otimizando o uso dos nutrientes (Sánchez-Vázquez et al., 2001) e, consequentemente, melhorando o crescimento do animal (Prieto-Guevara et al., 2015).

Sabendo que a alimentação contribui em grande parte, com os custos de produção, o conhecimento sobre a ritimicidade do apetite em peixes e o melhor aproveitamento dos

nutrientes é de grande relevância para os produtores, pois pode determinar o melhor horário de alimentação, minimizando desperdícios (López-Olmeda e Sánchez-Vázquez, 2009).

2.5 Influência dos ciclos de temperatura nos ritmos biológicos e desenvolvimento larvário

A temperatura é reconhecida como um dos fatores abióticos mais importantes que afetam a fisiologia (Martinez et al., 1996), comportamento, distribuição de organismos aquáticos (Brett, 1971), desenvolvimento embrionário (Kimmel et al., 1995; Schroter et al., 2008). Fry (1947) descreveu cinco efeitos principais que a temperatura causa nos peixes, em relação aos processos fisiológicos: controle, mascaramento, limitação, direção e atuando como um agente letal. Em relação a este último, temperaturas além dos limites ótimos de uma determinada espécie de peixe, podem influenciar na saúde, aumentando a taxa metabólica, o consumo de oxigênio e a virulência de patógenos.

Alguns estudos já demonstraram que a elevação da temperatura pode ocasionar um aumento de 25% na taxa de desenvolvimento dos embriões (Delaunay et al., 2000), além de diminuir o tempo de eclosão (Dekens et al., 2003). Como citado anteriormente, o aumento da temperatura da água também leva a um aumento no metabolismo, maior conversão alimentar e, consequentemente, maiores taxas de crescimento (Fauconneau, 1985; Blier et al., 1997; Koumoundouros et al., 2002).

Deane e Woo (2009) demonstraram que o crescimento dos peixes, induzido por altas temperaturas, pode ser medido pela ação da temperatura na produção do hormônio do crescimento (GH), já que seus níveis são aumentados com o aumento da temperatura. Os autores observaram que os níveis de GH estavam mais elevados nos peixes durante o verão, sendo assim influenciado pela variação sazonal.

No ambiente natural, oscilações diárias de temperatura (termociclos) são geradas na água devido à presença ou ausência de radiação solar (Villamizar et al., 2011). Assim, a temperatura exibe ciclos ao longo do ciclo claro/escuro, sendo que a fase de temperatura mais alta (termofase) coincide com o dia e a fase de temperatura mais baixa (criofase) coincide com a noite (López-Olmeda e Sánchez-Vázquez, 2011).

Alguns estudos vêm demostrando a capacidade de sincronização dos termociclos com as expressões de genes, atividade locomotora e eclosão de ovos (Lahiri et al., 2005; Lopez-Olmeda et al., 2006; Villamizar et al., 2012).

Blanco-Vives et al. (2010) demostraram a influência de termociclos no crescimento,

malformações da mandíbula, saco vitelínico e metamorfose em larvas de linguado (*Solea senegalensis*). Os autores observaram que numa condição de termociclo semelhante às condições naturais as larvas apresentaram maior desenvolvimento e menor porcentagem de deformidades, quando comparadas com larvas mantidas em temperatura constante. Além disso, os animais mantidos em termociclo iniciaram seus processos de metamorfose mais cedo.

Villamizar et al. (2012) observaram resultados semelhantes, ao aplicarem termociclos diários no desenvolvimento larval do zebrafish (*Danio rerio*), onde encontraram maior comprimento larval, maior taxa de eclosão, maior sobrevivência e menor taxa de deformidade.

A temperatura influencia uma série de características do animal, sobretudo no período larval, onde estão mais susceptíveis a mudanças de temperatura (Rombough et al., 1997). Os estudos com termociclos vêm demostrando benefícios no desenvolvimento das larvas de peixes, porém, ainda são escassos os trabalhos com este tema. Portanto, é necessário dar continuidade às pesquisas relacionadas com a influência de termociclos no período larval de peixes.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar os efeitos da temperatura no metabolismo e fisiologia digestiva de larvas de tilápia (*Oreochromis niloticus*).

3.2 Objetivos específicos

- Avaliar o crescimento de larvas de tilápia alimentadas e mantidas em temperaturas distintas;
- Avaliar, em um ciclo diário, os ritmos diários de expressão gênica de enzimas relacionadas à digestão de proteína, lipídios e carboidratos, em larvas de tilápia mantidas em diferentes temperaturas;
- Avaliar, em um ciclo diário, os ritmos diários de expressão gênica de enzimas relacionadas ao metabolismo de proteína, lipídios e carboidratos, em larvas de tilápia mantidas em diferentes temperaturas;
- Avaliar, em um ciclo diário, os ritmos diários de expressão gênica do

neuropeptídio Y (NPY) e de colecistoquinina (CCK) em larvas de tilápia mantidas em diferentes temperaturas.

4. REFERENCIAS BIBLIOGRÁFICAS

ARAÚJO, J. F.; MARQUES, N. (2002). Cronobiologia: uma multidisciplinaridade necessária. Margem, São Paulo, P. 95-112.

AYASHI, C., BOSCOLO, W. R., SOARES, C. M., MEURER, F. (2002). Exigência de proteína digestível para larvas de tilápia do Nilo (Oreochromis niloticus) durante a reversão sexual. Revista Brasileira de Zootecnia, 31(2), 823-828.

BALDISSEROTTO, B. (2002). Fisiologia de peixes aplicada à piscicultura. Santa Maria: UFSM.

BALDISSEROTTO, B., CYRINO, J. E. P., URBINATI, E. C. (2014). Biologia e fisiologia de peixes neotropicais de água doce.

BLIER, P. U., PELLETIER, D., DUTIL, J. D. (1997). Does aerobic capacity set a 33ficial33 fish growth rate. Reviews in Fisheries Science, 5(4), 323-340.

BOUGATEF, A. (2013). Trypsins from fish processing waste: characteristics and biotechnological applications–comprehensive review. Journal of cleaner production, 57, 257- 265.

BRETT, J. R. (1971). Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerkd*). American zoologist, 11(1), 99-113.

BROMAGE, N.; PORTER, M.; RANDALL, C. (2001). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture, 197, 63-98.

CAPERL, J.; LOZANO, R..; MARTÍNEZ-ZAPATER, J. M..; JARILLO, J. A. (2003). Ritmos y relojes circadianos de las plantas. Ecosistemas, 12, 3-9.

CHAMPE, P. C., HARVEY, R. A., FERRIER, D. R. (2006). Bioquímica ilustrada. 3ª. Edição

Porto Alegre: Artmed Editora. Chronobiology International, 23, 537–550.

COMPERATORE, C. A., STEPHAN, F. K. (1987). Entrainment of duodenal activity to periodic feeding. Journal of biological rhythms, 2(3), 227-242.

CYMBOROWSKI & INKULCZYKOWSKA, E., POPEK, W., KAPOOR, B. G. (Eds.). (2010). Biological clock in fish. CRC Press.

DEANE, E. E., WOO, N. Y. (2009). Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. Reviews in fish biology and fisheries, 19(1), 97-120.

DEKENS, M.P.S., SANTORIELLO, C., VALLONE, D., GRASSI, G., WHITMORE, D., FOULKES, N.S. (2003). Light regulates the cell cycle in zebrafish. Current Biology, 13, 2051– 2057.

DELAUNAY, F., THISSE, C., MARCHAND, O., LAUDET, V., THISSE, B. (2000). An inherited functional circadian clock in zebrafish embryos. Science 289, 297–300.

DUTTA, H.(1994). Growth in fishes. Gerontology, 40: 97–112.

FALCON, J. (1999). Cellular circadian clocks in the pineal. Prog. Neurobiol., 58, 121-16.

FALCON, J.; BESSEAU, L.; SAUZET, S.; BOEUF, G. (2007). Melatonin effects on the hypothalamo–pituitary axis in fish. Trends Endocrinology Metabolism, 18, 81-88.

FALCON, J.; MIGAUD, H.; MUNOZ-CUETO, J.A.; CARRILLO, M. (2010). Current knowledge on the melatonin system in teleost fish. General and Comparative Endocrinology, 165, 469-482.

FAUCONNEAU, B. (1985). Protein synthesis and protein deposition in fish. Nutrition and feeding in fish, 17-45.

Food, F. A. O. (2018). Agriculture Organization of the United Nations. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition fo

all. Rome. 200 pp.

FRY, F. E. J. (1971). The effect of environmental 35ficial35sm the physiology 35ficial. Fish physiology, 1-98.

FURNÉ, M., HIDALGO, M.C., LOPEZ, A., GARCIA-GALLEGO, M., MORALES, A.E., DOMEZAIN, A., DOMEZAINE, J., SANZ, A. (2005). Digestive enzyme activities in Adriatic sturgeon Acipenser naccarii and 35empera trout Oncorhynchus mykiss, A comparative study.Aquaculture 250, 391– 398.

GONÇALVES, R. C.; MARQUES, M. D. (2012) Ritmos de populações: o caso das abelhas sem ferrão. Revista de Biología. 9, 53–57.

HERRERO, M. J.; MADRID, J. A.; SÁNCHEZ-VÁZQUEZ, F. J. (2009). Entrainment to Light of Circadian Activity Rhythms in Tench (*Tinca tinca*). Chronobiol. Int., 20, 1001-1017.

HOAR, W.S., RANDALL, D.J. (Eds.), Fish Physiology—Volume IV Environmental

JOBLING, M. (1995). Simple 35ficial for the assessment of the influences of social environment on growth performance, exemplified by studies on Arctic charr. Aquaculture International, 3(1), 60-65.

JOBLING, M. (1988). A review of the physiological and nutritional energetics of cod (*Gadus morhua*) L., with particular reference to growth under farmed conditions. Aquaculture 70: 1- 19.

KIMMEL, C. B., BALLARD, W. W., KIMMEL, S. R., ULLMANN, B., SCHILLING, T. F. (1995). Stages of embryonic development of the zebrafish. Developmental dynamics, 203(3), 253-310.

KITAGAWA, A. T.; COSTA, L. S.; PAULINO, R. R.; ET AL. (2015). Feeding behavior and the effect of photoperiod on the performance and hematological parameters of the pacamã catfish (Lophiosilurus alexandri). Applied Animal Behaviour Science, 171, 211-218.

KOUMOUNDOUROS, G., SFAKIANAKIS, D. G., DIVANACH, P., & KENTOURI, M. (2002). Effect of 36emperatura on swimming performance of sea bass juveniles. Journal of Fish Biology, 60(4), 923-932.

KULCZYKOWSKA, E.; POPEK, W.; KAPOOR, B.G. Biological clock in fish. New York: CRC Press, 2010. P.280.

LAHIRI, K., VALLONE, D., GONDI, S.B., SANTORIELLO, C., DICKMEIS, T., FOULKES, N.S., 2005. Temperature regulates transcription in the zebrafish circadian clock. PloS Biol. 3, 351.

LAUFF, M., HOFER, R. (1984). Proteolytic enzymes in fish development and the importance of dietary enzymes. Aquaculture, 37(4), 335-346.

LAZADO, C.C.; PEDERSEN, P.B.; NGUYEN, H.Q.; LUND, I. (2017). Rhythmicity and plasticity of digestive physiology in a euryhaline teleost fish, permit (Trachinotus falcatus). Comp. Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology Special Issues, 212, 107-116.

LEHNINGER, AL, NELSON, DL, COX, MM (2006). Princípios de Bioquímica, 4. Edição. Sarvier, São Paulo.

LÓPEZ-OLMEDA, J. F., TARTAGLIONE, E. V., DE LA IGLESIA, H. O., SÁNCHEZ-

VÁZQUEZ, F. J. (2010). Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under 36ficial36s lighting and feeding conditions. Chronobiology international, 27(7), 1380-1400.

LÓPEZ-OLMEDA, J. F.; SÁNCHEZ-VÁZQUEZ, F. J. (2009). Zebrafish temperature selection and synchronization of locomotor activity circadian rhythm to ahemeral cycles of light and temperature. Chronobiology International, 26, 200-18.

LÓPEZ-OLMEDA, J. F., MADRID, J. A., SÁNCHEZ-VÁZQUEZ, F. J. (2006). Light and temperature cycles as zeitgebers of zebrafish (Danio rerio) circadian activity rhythms. Chronobiology international, 23(3), 537-550.

LÓPEZ-OLMEDA, J. F., LÓPEZ-GARCÍA, I., SÁNCHEZ-MUROS, M. J., BLANCO-VIVES, B., APARICIO, R., SÁNCHEZ-VÁZQUEZ, F. J. (2012b). Daily rhythms of digestive physiology, 37ficial37smo and behaviour in the European eel (Anguilla anguilla). Aquaculture international, 20(6), 1085-1096.

LÓPEZ-OLMEDA, J. F., NOBLE, C., SÁNCHEZ-VÁZQUEZ, F. J. (2012[a]). Does feeding time affect fish welfare?. Fish physiology and biochemistry, 38(1), 143-152.

MAITRA, S. K., CHATTORAJ, A., MUKHERJEE, S., MONIRUZZAMAN, M. (2013). Melatonin: a 37ficial candidate in the regulation of fish oocyte growth and maturation. General and comparative endocrinology, 181, 215-222.

MALIK, A., RUDOLPH, R., SÖHLING, B. (2005). Use of enhanced green fluorescent protein to determine pepsin at high sensitivity. Analytical biochemistry, 340(2), 252-258.

MARTINEZ-PALACIOS, C. A., CHAVEZ-SANCHEZ, M. C., ROSS, L. G. (1996). The effects of water temperature on food intake, growth and body composition of Cichlasoma urophthalmus (Günther) juveniles. Aquaculture Research, 27(6), 455-461.

MEURER, F.; HAYASHI, C.; BOSCOLO, R. W. (2003). Digestibilidade Aparente de Alguns Alimentos Proteicos pela Tilápia do Nilo (Oreochromis niloticus). Revista Brasileira de zootecnia, 32, 1801-1809.

MISTLBERGER, R. E. (1994). Circadian food-anticipatory activity: formal models and physiological mechanisms. Neuroscience & Biobehavioral Reviews, 18(2), 171-195.

MONTOYA, A., LÓPEZ-OLMEDA, J. F., GARAYZAR, A. B. S., SÁNCHEZ-VÁZQUEZ, F. J. (2010b). Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in Gilthead seabream (*Sparus aurata*) under a light– dark cycle. Physiology & behavior, 101(1), 101-107.

MONTOYA, A., LÓPEZ-OLMEDA, J. F., YÚFERA, M., SÁNCHEZ-MUROS, M. J., SÁNCHEZ-VÁZQUEZ, F. J. (2010[a]). Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (*Sparus aurata*). Aquaculture, 306(1-4), 315- 321.

MORGAN, E. (2004). Ecological significance of biological clocks. Biological Rhythm Research, 35(1-2), 3-12.

MOTTA, V. T. (2003). Bioquímica básica. São Paulo-SP: Autolab.

NANDLAL, S.; PICKERING, T. Tilapia fish farming in Pacific Island countries, 1.Ed. Fiji Islands, 2004. 02p.

NEJDI, A., GUASTAVINO, J. M., LALONDE, R. (1996). Effects of the light-dark cycle on a 38fici tank social interaction test in mice. Physiology & behavior, 59(1), 45-47.

NOGUEIRA, A. C; RODRIGUES, T. (2007). Criação de tilápias em tanques-rede. –Sebrae Bahia, Salvador, 09p.

PEREZ-CASANOVA, J. C., MURRAY, H. M., GALLANT, J. W., ROSS, N. W., DOUGLAS, S. E., JOHNSON, S. C. (2006). Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture, 251(2-4), 377- 401.

PRIETO-GUEVARA, M. J., SILVA, R. F., COSTA, L. S., PEREIRA, R. T., ROSA, P. V. (2015). Effect of fixed feeding time on growth, body composition, and hepatic histology of hybrid catfish (Pseudoplatystoma reticulatum x Leiarius marmoratus) fed with carbohydrates and lipids ratios. *Revista Colombiana de Ciencias Pecuarias*, *28*(1), 83-92.

ROENNEBERG, T., MERROW, M. (2005). Circadian clocks—the fall and rise of physiology. Nature Reviews Molecular Cell Biology, *6*(12), 965-971.

ROMBOUGH, P. J. (1997, January). The effects of temperature on embryonic and larval development. In Seminar Series-Society For Experimental Biology (Vol. 61, pp. 177-224). Cambridge University Press.

SAHA, S., SINGH, K. M., & GUPTA, B. B. P. (2019). Melatonin synthesis and clock gene regulation in the pineal organ of teleost fish compared to mammals: Similarities and differences. General and comparative Endocrinology, 279, 27-34.

SÁNCHEZ-VÁZQUEZ, F. J., MADRID, J. A. (2001). Feeding anticipatory activity. Food intake in fish, 216-232.

SÁNCHEZ-VÁZQUEZ, F. J., ARANDA, A., MADRID, J. A. (2001). Differential effects of meal size and food energy density on feeding entrainment in goldfish. Journal of biological rhythms, *16*(1), 58-65.

SCHRÖTER, C., HERRGEN, L., CARDONA, A., BROUHARD, G. J., FELDMAN, B., OATES, A. C. (2008). Dynamics of zebrafish somitogenesis. Developmental dynamics: an 39ficial publication of the American Association of Anatomists, 237(3), 545-553.

SCHULZ, U. H., LEUCHTENBERGER, C. (2006). Activity patterns of South American silver catfish (*Rhamdia quelen*). Brazilian Journal of Biology, *66*(2ª), 565-574.

SCHWARZ, K. K., FURUYA, W. M., NATALI, M. R. M., GAUDEZI, M. C., LIMA, P. A. G. D. (2011). Mananoligossacarídeo em dietas para larvas de tilápia. Revista Brasileira de Zootecnia, 40(12), 2634-2640.

TAVARES-DIAS, M.; MARIANO, W. Aquicultura no Brasil: novas perspectivas. São Carlos: Pedro & João Editores, 2015.

VERA, L. M., DE PEDRO, N., GOMEZ-MILAN, E., DELGADO, M. J., SANCHEZ-MUROS, M. J., MADRID, J. A., SANCHEZ-VAZQUEZ, F. J. (2007). Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiology & behavior, 90(2-3), 518-524.

VILLAMIZAR, N., RIBAS, L., PIFERRER, F., VERA, L.M., SÁNCHEZ-VÁZQUEZ, F.J., (2012). Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. PloS ONE 7(12), 52153.

VILLAMIZAR, N., BLANCO-VIVES, B., MIGAUD, H., DAVIE, A., CARBONI, S., SANCHEZ-VAZQUEZ, F. J. (2011). Effects of light during early larval development of some aquacultured teleosts: a review. Aquaculture, *315*(1-2), 86-94.

VOLPATO, G.L.; TRAJANO, E. Biological Rhythms- Fish physiology. San Diego: Elsevier, 2006. P.101-153.

WULUND, L., REDDY, A. B. (2015). A brief history of circadian time: The emergence of redox oscillations as a novel component of biological rhythms. Perspectives in Science, *6*, 27- 37.

CAPÍTULO 1

Effects of temperature regime on growth and daily rhythms of digestive factors in Nile tilápia *(Oreochromis niloticus)* larvae

Artigo publicado no periódico Aquaculture (Anexo)

Amanda Hastenreiter Espírito Santo, Gonzalo de Alba, Yane da Silva Reis, Leandro Santos Costa, Francisco Javier Sánchez-Vázquez, Ronald Kennedy Luz, Paula Adriane Perez Ribeiro, Jose Fernando López-Olmeda.

https://doi.org/10.1016/j.aquaculture.2020.735545

Abstract

Environmental factors such as light and temperature present cyclic variations during the day. High temperatures occur during the light phase, whereas low temperatures take place at night (*i.e.* a daily thermocycle). Although applying thermocycles to fish larvae improves growth in some species, nothing is known about the impact during the early development of the digestive system. The aim of the present research was to investigate the effect of different temperature regimes, cycling *versus* constant, on the daily rhythms of digestive factors and growth of Nile tilapia (*Oreochromis niloticus*) larvae. For this purpose, fertilized eggs (stage 1) were divided into two groups: one under a thermocycle (TC) of 31 °C:25 °C day:night and another group at constant temperature of 28 °C (CTE). Photoperiod was set at 12:12 h light/dark cycle. Larvae length was measured on 4-, 8- and 13-days post- fertilization (dpf) and samples were collected every 4 h during a 24-h cycle on these days. The expression levels of *pepsinogen*, *chymotrypsinogen*, *trypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck* were analyzed by qPCR. The results showed that growth was greater when larvae were reared at TC than at CTE. Moreover, on 13 dpf, most analyzed genes (*chymotrypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck*) displayed daily rhythms in the TC group but not in CTE, with most acrophases located around mealtime. These rhythms might explain the higher growth rate observed in the TC larvae due to improved synchronization of feed digestion and utilization. These findings could be useful for improving rearing protocols used in tilapia larviculture and enhancing production performance.

Keywords: Biological clock Larviculture. Fish development. Digestive function Endocrine system

1. Introduction

The natural environment presents cyclic and predictable variations in geophysical variables, such as light and temperature. These cyclic oscillations have influenced evolution and selected the occurrence of biological clocks in most living organisms. Biological rhythms confer adaptive advantages as organisms can time their physiological pro- cesses to occur at specific moments when effectiveness is the greatest (DeCoursey, 2004). Among the environmental variables that synchro- nize biological rhythms, light has been commonly regarded as the most important but other factors, such as temperature and feeding, play an important role, especially in ectothermic animals like fish (López- Olmeda, 2017). Temperature has a strong influence on fish behavior and physiology (Brett, 1971) as it affects processes like development, locomotion, sex differentiation, reproduction and survival (Bennett and Beitinger, 1997; Ospina-Álvarez and Piferrer, 2008; Pankhurst and King, 2010; López- Olmeda and Sánchez-Vázquez, 2011). In the natural environment, daily temperature oscillations (thermocycles) are generated in the water due to the presence or absence of solar infrared radiation (Villamizar et al., 2011). Hence temperature displays cycles alongside the light/dark cycle, with the higher temperature phase (thermophase) coinciding with day and the lower temperature phase (cryophase) coinciding with night (López-Olmeda and Sánchez-Vázquez, 2011). However, while most fish undergo thermocycles in the wild, their effects on fish biology, especially during early development, have received little attention to date (Schaefer and Ryan, 2006; Villamizar et al., 2011). Thermocycles during larval development have been reported to in- crease survival and growth, lower the incidence of malformations and modify the sex ratio (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018).

Nile tilapia (*Oreochromis niloticus*) is a species that tolerates a rela- tively broad range of temperatures and is subjected to wide tempera- ture variations in the wild, from temperate to warm waters (Omondi et al., 2014; Ndiwa et al., 2016). For instance, in one of its natural environments, the lakes of the Great Rift Valley in Africa, air tem- perature shows wide daily fluctuations (Crul, 1995), which generate daily variations in water temperature of several degrees of amplitude, *i.e.* 24–30 °C (min–max) (Patterson and Wilson, 1995). Moreover, al- though temperature is controlled in many culture systems used for ti- lapia (Boyd, 2004), similar variations may occur in extensive open systems such as ponds or net cages located in rivers,

where water temperature can display daily variations of up to 5–6 °C (Culberson and Piedrahita, 1996; Jomori et al., 2003; Lopes and Henry-Silva, 2014). However, despite the importance of the variations in water temperature in its natural habitat, this topic has received little consideration in previous studies on tilapia development (Azaza et al., 2010)

One of the most important factors to affect the efficiency of food conversion into nutrients is the availability of the digestive enzymes involved in this process (Jobling, 1995; Perez-Casanova et al., 2006). The maximum fish growth rate, especially during early development, strongly depends on digestive capacity (Blier et al., 1997). Thus re- search on the ontogeny of digestive enzymes in fish can elucidate cer- tain aspects of their nutritional physiology and help to solve nutritional challenges in larviculture (Furné et al., 2005). In fish, studies on the ontogeny of the digestive function at the molecular level are scarce. In addition, studies on the existence of rhythms of digestive factors during development and how the environment modulates these rhythms are lacking (Rønnestad et al., 2013; Yúfera et al., 2018). Studying the ex- istence of daily rhythms in larval physiology is also crucial because most research has usually focused only on one single daily sample, neglecting daily oscillations (Yúfera et al., 2018). To date, the existence and ontogeny of these rhythms in fish larvae have only been reported in two marine species: gilthead sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016).

The larviculture of Nile tilapia offers some advantages, such as using formulated diets from the start of exogenous feeding (Luz et al., 2012). However, for this species, more in-depth knowledge about the digestive physiology in the larval stage is necessary (De Moura Pereira et al., 2019). To date, research into the ontogeny of digestive factors during larval development is scarce, and only a few reports have addressed this topic (Tengjaroenkul et al., 2002; Qiang et al., 2017; De Moura Pereira et al., 2019; Silva et al., 2019). Daily rhythms in clock gene expression and factors from the endocrine axis have been reported in adult Nile tilapia (Costa et al., 2016a, 2016b; de Alba et al., 2019), but still nothing is known about the existence of the daily rhythms in digestive factors for tilapia larvae.

Therefore, the aim of the present study was to evaluate the effects of two different temperature regimes, a daily thermocycle *versus* constant temperature, applied on the first days of Nile tilapia development, on the growth and daily rhythms of different digestive physiology para- meters. These parameters involved the expression of genes that encode enzymes for protein (*pepsinogen*, *chymotrypsinogen* and *trypsinogen*), lipid (*lipase*) and carbohydrate

digestion (*maltase* and *isomaltase*), and the endocrine control of food intake and digestion (*neuropeptide Y*, *npy*, and *cholecystokinin*, *cck*).

2. Materials and methods

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia (Spain). Fish were reared following the Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish leg- islation (RD 53/2013 and Law 32/2007) for the use of laboratory an- imals. In addition, the protocols were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

2.1 Animals and experimental design

Male and female Nile tilapia (Oreochromis niloticus) adults were obtained from a local fish farm (Tilamur S.A., Murcia, Spain). Animals were kept in 300-l tanks connected to a recirculation system, equipped with biological and mechanical filters. The photoperiod was set at 12:12 h light/dark (LD) cycle with lights on at 09:00 h. The water temperature was maintained controlled at 28 ± 0.5 °C. Adult tilapias were fed a commercial diet (D-4 AlternaBasic 2P, Skretting, Spain) with 36% crude protein (CP) 3 times a day until apparent satiety.

After acclimation, tilapia breeders were induced with human Chorionic Gonadotropin hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013). Briefly, females were intraperitoneally (i.p.) injected with 1500 IU/kg of body weight and males with 500 IU/kg. The administration for females was divided into two i.p. injections: the first consisted of 500 IU/kg injected at the end of the light phase and the second one (1000 IU/kg) was applied 12 h after the first dose (at the beginning of the light phase on the next day). Males received a single i.p. injection at the same time as the second dose for females. After hCG administration, animals were placed to- gether. After 24 h, sperm and eggs were collected by stripping and in vitro fertilization was performed (Fernandes et al., 2013). This proce- dure allowed us to immediately obtain fertilized eggs, which were used in the experiments.

Fertilized eggs were obtained from five different groups of tilapia breeders (3 females and 5–6 males per group). Then eggs at stage 1 (Fujimura and Okada, 2007) were pooled together and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (150–200

eggs per incubator, 6 incubators per group were used) in two distinct sys- tems with different temperature regimes: one system with a daily thermocycle (TC) of 31 °C:25 °C, and another with a constant tem- perature (CTE) of 28 °C. Both systems were recirculating water systems that were connected to mechanical and biological filters. The photo- period was set at 12:12 LD, with lights on at 09:00 h. By convention, in chronobiological studies, the time of light onset is set as Zeitgeber (synchronizer) time 0 h (ZT0 h), in order to standardize time points regardless local time, facilitating reproducibility (Guerra-Santos et al., 2017). Hence, light onset at 9:00 h corresponds to ZT 0 h and light offset at 21:00 h corresponds to ZT 12 h. In the TC system, the ther- mophase (high temperature phase) of 31 °C coincided with the light phase, whereas the cryophase (low temperature) of 25 °C coincided with the dark phase (Suppl. Fig. 1A). Temperature difference between the thermo- and the cryophase was selected following previous litera- ture and the normal daily ranges experienced by tilapia in the wild (Patterson and Wilson, 1995; Schaefer and Ryan, 2006; Villamizar et al., 2012; Ndiwa et al., 2016). The average water temperature in the TC system throughout the day was 28.0 °C. Thus the larvae reared in this system were subjected to the same degree days as the animals reared in the CTE group, at an average water temperature of 28.0 ± 0.3 °C (mean ± SD). In the TC system, water temperature was modified by water heaters (Askoll, Povolaro, Italy) and coolers (Aqua- Medic 1500, Titan GmbH, Bissendorf, Germany), controlled by elec- tronic timers (Bachmann GmbH & Co, Stuttgart, Germany). Water temperature was recorded continuously throughout the experiment by an underwater data recorder (HOBO pendant, Onset Computer Cor- poration, Bourne, MA, USA). Hatching time (day) and rate were re- corded for posterior analysis on the influence of the thermal regime on these parameters. Larvae were reared in incubators until 7 days post fertilization (dpf), when they were transferred to 9-l tanks connected to the same temperature system. Density in the tanks was of approxi- mately 10 larvae/l, and a total of ten 9-l tanks were used in the ex- periments (5 tanks per temperature treatment). Exogenous feeding began on 7 dpf. Larvae were fed a semi-purified diet containing 42% CP and 4100 Kcal/kg, formulated as described elsewhere (Silva et al., 2019). Larvae were fed in excess 4 times a day in the first half of the light phase (ZT 1, ZT 3, ZT 5 and ZT 7 h) (Suppl. Fig. 1B). Tilapia embryo/larvae were maintained under the experimental conditions from 0 to 13 dpf.

A total of 1014 tilapia larvae were used in the experiments: 864 larvae for qPCR analyses and 150 larvae for total length measures. On 4, 8 and 13 dpf, whole larvae samples were collected every 4 h during a 24-h cycle at the following time points: ZT 2, ZT 6, ZT 10, ZT 14, ZT 18 and ZT 22 h (Suppl. Fig. 1B). Larvae were pooled for each replicate and six replicates

(n = 6) were collected for each group, time point and day. Larvae were stored in 1.5 ml tubes and immediately frozen and stored at −80 °C until analyzed. The number of larvae used in the pool dif- fered depending on the sampling day: 5 larvae/pool on 4 dpf; 4 larvae/ pool on 8 dpf; 3 larvae/pool on 13 dpf. The larvae collected on 8 and 13 dpf were fed on the sampling day.

2.2 Larval growth

The growth of the tilapia larvae in both temperature groups (TC and CTE) was evaluated on 4, 8 and 13 dpf. For this purpose, 75 larvae were used for each treatment, and 25 larvae were used for each measurement day. Larvae were removed from the aquarium and transferred to a Petri dish. Then each larvae was photographed using a binocular (Leica EZ4 HD, Leica Microsystems GmbH, Wetzlar, Germany) with an in- corporated digital camera. Photos were stored in a computer and ana- lyzed later with the ImageJ image processing software (Abramoff et al., 2004). Larvae were measured longitudinally by considering standard length (Suppl. Fig. 2).

2.3 Real-time RT-PCR analysis

Larvae pools were transferred to sterile tubes and homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) fol- lowing the manufacturer's instructions, using a tissue homogenizer for mechanical homogenization (TissueLyser LT, Qiagen, Hilden, Germany). RNA was dissolved in DEPC water (Invitrogen, Carlsbad, USA) and RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Total RNA (1 µg) was first treated with 1 U of DNase (Dnase I, Thermo Fisher), followed by retrotranscription using a commercial kit (qScript cDNA Synthesis Kit, Quantabio, Beverly, USA). The quantitative PCR (qPCR) reactions were performed by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). All the samples were run in duplicate and the qPCR reactions were performed in a final volume of 20 µl. The quantitative PCR analyses were performed in a light thermocycler (7500 RT-PCR system, Applied Biosystems) following this protocol: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were run after amplification to ensure that only one DNA species was amplified. All the primer sequences (Table 1) were designed with the Primer 3 plus software (Untergasser et al., 2012). The relative ampli- fication efficiencies of all the genes were analyzed by cDNA dilution curves and were found to be similar for all the genes. Primer con- centrations were determined by means of a primer dilution curve. The primers for

chymotrypsinogen, pepsinogen, lipase, npy, maltase and βactin were added at a reaction concentration of 200 nM. The primers for trypsinogen, isomaltase and cck were added at a final concentration of 400 nM. The relative expression of all the genes was calculated by the 2–ΔΔCt method (Livak and Schmittgen, 2001). The reference gene, βactin, was selected after verifying that its coefficient of variation (CV) was lower than 5% and was used in the first normalization. The sample with the lowest expression value within each gene, tissue and group was used as the reference for the second normalization.

2.4 Data analysis

All the results are expressed as mean ± SEM. The significance threshold was set at $\alpha = 0.05$ for all the tests. The SPSS software (v. 19.0, IBM, Armonk, USA) was used to detect any statistically significant differences between groups and time points. Normality of the data was previously assessed by the Kolmogorov-Smirnov test and homogeneity of variance was also verified using Levene's test. Data were subjected to a two-way ANOVA, followed by Duncan's post hoc test, to check for statistically significant differences in gene expressions between groups (TC versus CTE) and sampling points (ZTs), and differences in length between groups and days post fertilization (4, 8 and 13 dpf). Data of hatching rate and hatching day were subjected to a Student's t-test and a Mann-Whitney U test, respectively.

The existence of significant rhythmicity was tested for all the genes by the Cosinor analysis, performed with the “EL TEMPS” software (v.1.179, Prof. Díez-Noguera, University of Barcelona, Spain). The Cosinor analysis is based on the least squares approach of time series data with a cosine function of a known period of type $Y = Mesor + Amplitude * \cos(2\pi(t\text{-}Acrophase)/Period)$. The Cosinor analysis also provides the statistical significance of the rhythm by an F- test of the variance, accounted for by the waveform versus a straight line of zero-amplitude (null hypothesis).

3. Results

3.1 Hatching timing and rate

The eggs presented a similar hatching rate in the two temperature rearing conditions (*t*-test, $p = .652$). Eggs at TC had a hatching rate of 49.6 ± 13.3% (mean ± SEM) whereas eggs at CTE presented a rate of 50.5 ± 11.6%. Regarding hatching time, all eggs hatched between 2 and 4 dpf, with most hatching events occurring at days 2 and 3. Eggs at TC hatched mainly

at 2 dpf (2.3 ± 0.3 dpf, mean ± SEM) whereas eggs at CTE hatched mainly at 3 dpf (3.3 ± 0.3 dpf), but these dif- ferences were not statistically significant (Mann-Whitney U test, $p = .114$).

3.2 Larval growth

The length was significantly influenced by the developmental stage (dpf) (two-way ANOVA, $p < .001$), the temperature regime in which tilapia embryo and larvae were raised ($p = .001$) and the interaction between these two factors ($p < .001$) (Fig. 1). In both temperature treatments, differences were observed between 4, 8 and 13 dpf, with larval length significantly increasing from one developmental stage to the following one (Fig. 1). In addition, significant differences were observed between groups at 13 dpf. By this day, the TC treatment larvae presented significantly higher length values (8.09 ± 0.07 mm, mean ± SEM) compared to those in the CTE treatment (7.77 ± 0.09 mm) (Fig. 1).

3.3 *Pepsinogen* expression

No daily rhythms (Cosinor, $p = .068–0.345$) were found for *pepsi- nogen* relative expression in the 4, 8 or 13 dpf tilapia larvae in either of the tested temperature regimes (Fig. 2). Statistically significant effects were observed for time at 4 dpf and group at 13 dpf (two-way ANOVA, $p = .001$ and 0.026, respectively) (Suppl Table 1). In addition, a sig- nificant effect in the interaction of both factors (time and group) was detected at 4 and 13 dpf ($p = .002$ and 0.041, respectively) (Fig. 2A and C). At 4 dpf, *pepsinogen* expression in TC at ZT 2 h was significantly higher than CTE at the same time and other time points in the TC group (Fig. 2A). In addition, on 13 dpf, *pepsinogen* levels in CTE at ZT 2 h were higher than TC at the same time and other time points in the CTE group (Fig. 2C).

3.4 *Chymotrypsinogen* expression

The relative expression of *chymotrypsinogen* displayed significant daily rhythms for the 4 dpf larvae maintained in the CTE group (Cosinor, $p = .018$), on 8dpf in both treatments ($p = .024$ and $p = .007$ for TC and CTE, respectively) and for the 13 dpf larvae maintained in TC ($p = .044$) (Fig. 3). In addition, the acrophases varied between the significant detected rhythms (Table 2). In the CTE group, acrophases were located in the dark phase at ZT 18:06 h and ZT 21:35 h for 4 and 8 dpf, respectively. In the TC group, the acrophase was located at the beginning of the dark phase on 8 dpf (ZT 14:02 h) and shifted to the beginning of the light

phase on 13 dpf (ZT 02:36 h). Moreover, *chy- motrypsinogen* expression in the 8 dpf larvae showed significant differ- ences depending on the groups (two-way ANOVA, $p = .002$) and in the interaction between time and group factors ($p = .007$) (Fig. 3B and Suppl Table 1). At 8 dpf, *chymotrypsinogen* in the TC group showed significant variations between time points, with higher expression at ZT 14 h than ZT 2, 6 and 22 h (Fig. 3B). The expression was also higher in TC at ZT 10 and 14 h compared with the CTE group (Fig. 3B).

3.5 *Trypsinogen* expression

The relative expression of trypsinogen in tilapia larvae showed sig- nificant daily rhythms in the larvae raised in TC on both 4 and 8 dpf (Cosinor, $p = .029$ and $p = .025$, respectively) (Fig. 4). The acrophase of *trypsinogen* for the 4 dpf TC was located at the beginning of the light phase (ZT 03:10 h) and shifted to the end of the light phase in the 8 dpf larvae (ZT 10:11 h) (Table 2). In addition, *trypsinogen* in the 4 dpf larvae displayed significant differences depending on the group (two-way ANOVA, $p = .002$), the time of the day ($p < .001$) and the interaction between these two factors ($p < .001$) (Fig. 4A and Suppl Table 1). At this stage, *pepsinogen* expression in TC at ZT 2 h was significantly higher than CTE at the same time and other time points in the TC group (Fig. 4A).

3.6 *Lipase* expression

Lipase expression only displayed significant daily rhythms in the tilapia maintained under the TC conditions on both 4 and 13 dpf (Cosinor, $p = .044$ and $p = .009$, respectively) (Fig. 5). The acrophases in these two stages occurred at similar times, in the first half of the light phase (ZT 03:54 h and 02:47 h on 4 and 13 dpf, respectively) (Table 2). Furthermore, lipase expression showed significant differences de- pending on the group at 8 dpf (two-way ANOVA, $p < .001$), depending on the time of the day at 4 and 8 dpf ($p = .010$ and 0.019, respectively), and in the interaction of both factors at 4 and 8 dpf ($p = .022$ and $p < .001$, respectively) (Fig. 5 and Suppl Table 1). On 4 dpf, *lipase* levels in the TC group peaked at ZT 2 h and were significantly higher than levels at ZT 10 and 18 h in the same group (Fig. 5A). At this stage, significant differences in lipase between TC and CTE were found at ZT 18 h (Fig. 5A). On 8 dpf, *lipase* expression was higher in the CTE group than the TC group at all time points except ZT 22 h (Fig. 5B). In ad- dition, *lipase* expression showed differences between time points within the CTE group at 8 dpf, with higher levels at ZT 2 h (Fig. 5B).

3.7 *Maltase* expression

The only significant daily rhythm in maltase expression was detected in the 13 dpf larvae from the TC group (Cosinor, p = .021) (Fig. 6). In these animals, maltase expression peaked around the middle of the dark phase, at ZT 17:27 h (Table 2). Moreover, statistically significant dif- ferences were found in maltase expression depending on the group factor at 8 dpf (two-way ANOVA, p = .008). At this stage, maltase ex- pression was higher in the CTE than in the TC group (Fig. 6B and Suppl Table 1).

3.8 Isomaltase expression

Significant daily rhythms of isomaltase were found at 13 dpf in both groups (Cosinor, p = .002 and p = .042 for TC and CTE, respectively) (Fig. 7). These rhythms presented opposite acrophases, with isomaltase expression peaking around the middle of the dark phase in the TC group (ZT 19:33 h) and about the middle of the light phase in the CTE group (ZT 05:28 h) (Table 2). Significant differences in isomaltase expression were observed depending on the group at 8 and 13 dpf (two-way ANOVA, p = .012 and 0.006, respectively), and in the interaction be- tween group and time factors (p = .001) (Fig. 7 and Suppl Table 1). The overall daily expression of isomaltase was higher in CTE than TC at 8 dpf. In addition, on 13 dpf, the TC group showed a higher isomaltase expression than CTE at ZT 18 and 22 h (Fig. 7C). The expression of isomaltase also showed differences between time points within the TC group at 13 dpf, with higher levels at ZT 18 and 22 h compared with the rest of sampling times (Fig. 7C)

3.9 *Npy* expression

Significant daily rhythms were found for npy expression in 4 dpf larvae maintained in CTE (Cosinor, p = .035) and the 13 dpf larvae from the TC group (p = .007) (Fig. 8). Acrophases were located in the middle of the dark phase (ZT 18:38 h) in the 4 dpf CTE larvae and in the first half of the light phase (ZT 03:00 h) in the 13 dpf TC larvae (Fig. 8A and C) (Table 2). The 13 dpf larvae presented significant differences in npy expression depending on the group (two-way ANOVA, p < .001), and in the interaction between group and time factors (p < .001) (Fig. 8C and Suppl Table 1). The CTE group showed a higher npy ex- pression than TC at ZT 2, 6 and 22 h (Fig. 8C). The expression of npy also showed differences between time points within the CTE group at 13 dpf, with higher levels at ZT 22 h compared with ZT 10, 14 and 18 h (Fig. 8C).

3.10 *Cck* expression

The only significant daily rhythm in cck expression was detected in the 13 dpf larvae from the TC group (Cosinor, p = .021) (Fig. 9). The acrophase of this rhythm was located in the first half of the light phase, close to the middle of the day (ZT 04:19 h) (Fig. 9C and Table 2). Statistically significant differences were found in cck expression de- pending on the group factor at 8 dpf (two-way ANOVA, p = .003) (Suppl Table 1). At this stage, the overall cck expression was higher in the TC group compared with CTE (Fig. 9B).

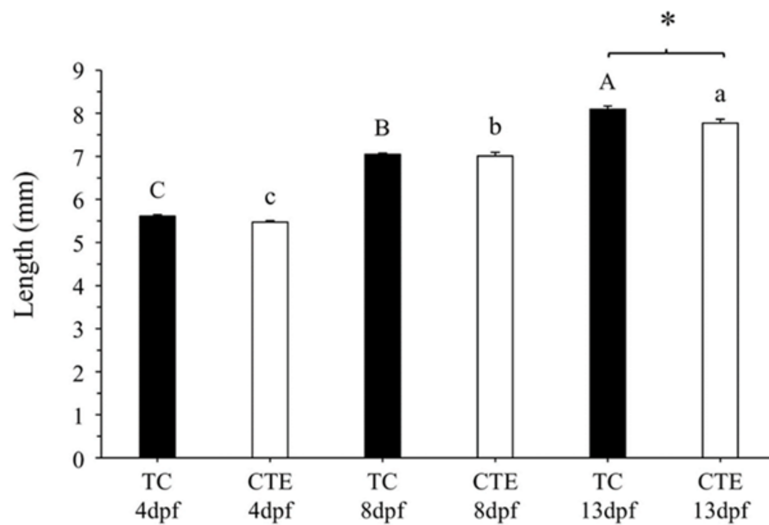


Fig. 1. Standard length (mm) of tilapia larvae on 4, 8 and 13 days post ferti- lization (dpf) at a 12:12LD cycle. Larvae were subjected to two different tem- perature regimes: a thermocycle (TC) of 31 °C:25 °C (black bars) or constant temperature (CTE) of 28 °C (white bars). Different upper case and lower case letters indicate statistically significant differences between developmental stages (dpf) within the TC or CTE groups, respectively (two-way ANOVA). The asterisk indicates significant differences between groups in the same dpf (two- way ANOVA). Data are represented as mean ± SEM; n = 25 replicates (larva) per point.

Table 1
Primer sequences used for the quantitative analysis by qPCR.

Gene	F/R	Sequence (5′-3′)	Amplification efficiency (%)	Ensembl/GenBank accession number
pepsinogen	F	TGACCAATGACGCTGACTTG	96.49	JQ043215.1
	R	GGAGGAACCGGTGTCAAAAATG		
chymotrypsinogen	F	TTCTGCCTTCGCTTCTCATC	101.95	ENSONIG00000003237
	R	TTCAACGCCATCTGCTACTG		
trypsinogen	F	AGTGCGCAAAGAACTCTGTG	101.65	AY510093.1
	R	AATGTTGTGCTCACCAAGGC		
lipase	F	CTACAACTGCTCCACCAGCA	95.17	NM_001279753.1
	R	GGTGTAGTCGGTGAGCCATT		
maltase	F	ACGGTGGAATCACAGGACTC	97.71	XM_005459498.4
	R	GAAGGCTGCTGATGTGTTCA		
isomaltase	F	GGATCATTCTTCTGGGACGA	103.98	XM_003441717.4
	R	AGGTTGTGCTGTGGGGTTAG		
cck	F	AGAAACTCCACGGCAAACAG	96.30	ENSONIG00000019439.1
	R	ACTCATACTCCTCTGCACTGC		
npy	F	ACACCCAACACTGCTTGAAG	102.21	ENSONIG00000004499
	R	TGTTGCACAGATGACGACTC		
βactin	F	TGGTGGGTATGGGTCAGAAAG	98	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA		

Table 2
Acrophase and statistical significance values of the genes subjected to Cosinor analysis.

Genes	dpf	Acrophase (ZT h)	
		TC CTE	
pepsinogen	4	–	–
	8	–	–
	13	–	–
chymotrypsinogen	4	–	18:06 ± 3:49*
	8	14:02 ± 3:56*	21:35 ± 3:11*
	13	2:36 ± 5:10*	–
trypsinogen	4	3:10 ± 4:52*	–
	8	10:11 ± 4:12*	–
	13	–	–
lipase	4	3:54 ± 5:05*	–
	8	–	–
	13	2:47 ± 3:16**	–
maltase	4	–	–
	8	–	–
	13	17:27 ± 4:03*	–
isomaltase	4	–	–
	8	–	–
	13	19:33 ± 2:08**	5:28 ± 4:09**
npy	4	–	18:38 ± 4:38*
	8	–	–
	13	3:00 ± 3:09**	–
cck	4	–	–
	8	–	–
	13	4:19 ± 3:57*	–

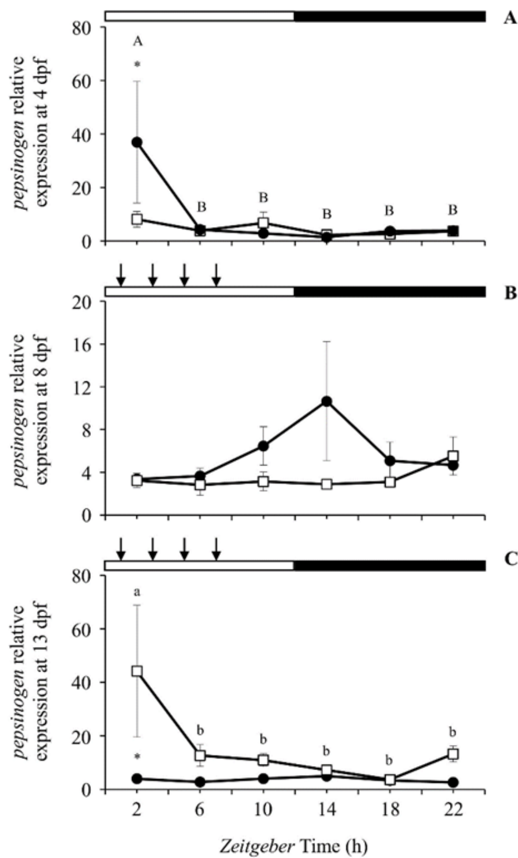


Fig. 2. Daily variations in the relative mRNA expression (fold change) of pep- sinogen analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertili- zation (dpf) at a 12:12 LD cycle. Larvae were subjected to two different tem- perature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, re- spectively, on the same graph (two-way ANOVA). The asterisks indicate sig- nificant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

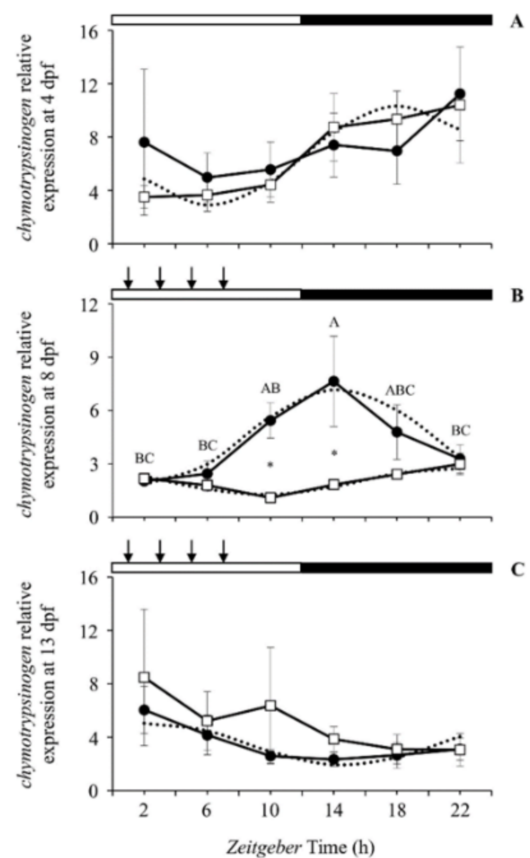


Fig. 3. Daily variations in the relative mRNA expression (fold change) of chy- motrypsinogen analyzed in tilapia larvae on 4 (A) and 13 (B) days post fertili- zation (dpf) at a 12:12 LD cycle. Larvae were subjected to two different tem-perature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinu- soidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

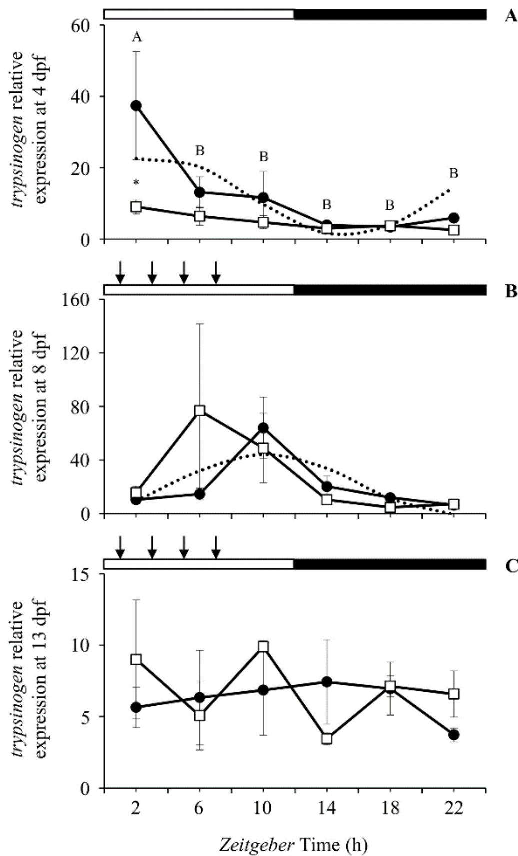


Fig. 4. Daily variations in the relative mRNA expression (fold change) of trypsinogen analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fer- tilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinu- soidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

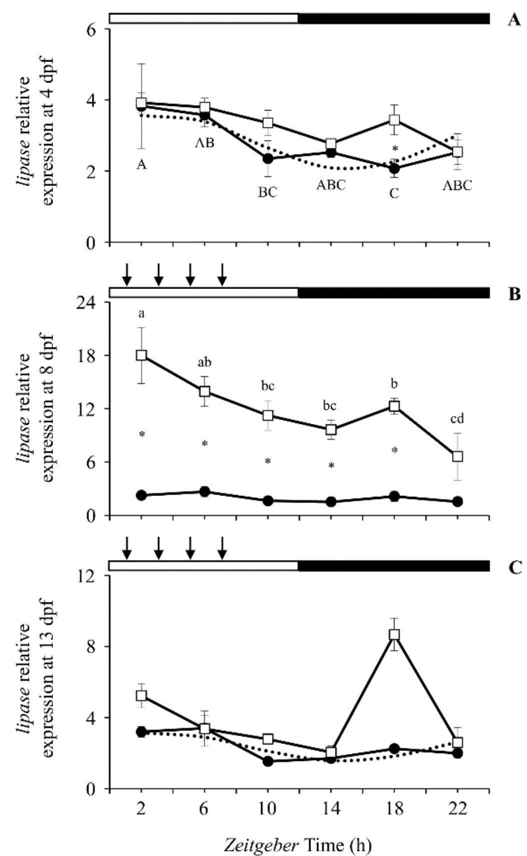
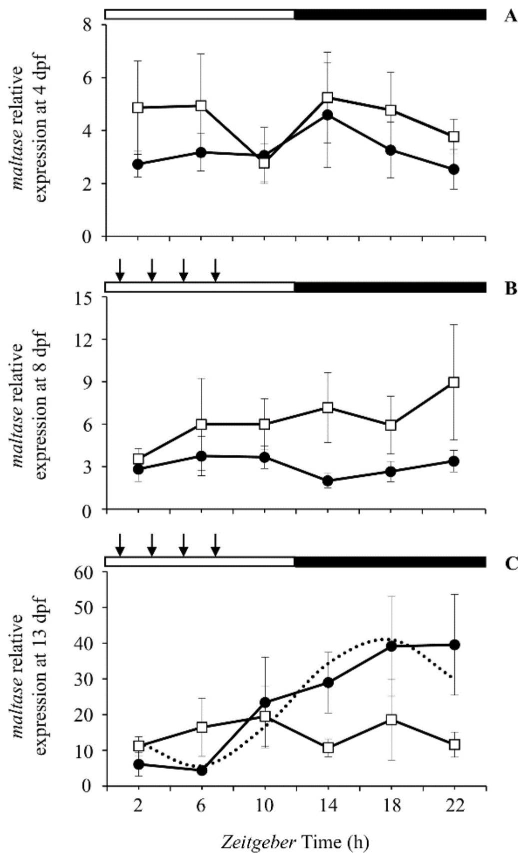


Fig. 5. Daily variations in the relative mRNA expression (fold change) of lipase analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks in- dicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.



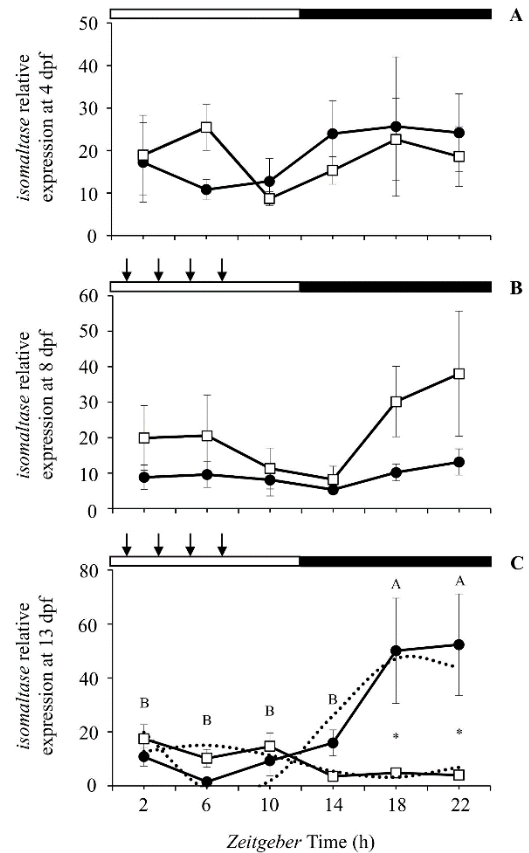


Fig. 6. Daily variations in the relative mRNA expression (fold change) of mal- tase analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

Fig. 7. Daily variations in the relative mRNA expression (fold change) of iso- maltase analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertili- zation (dpf) ata 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinu- soidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

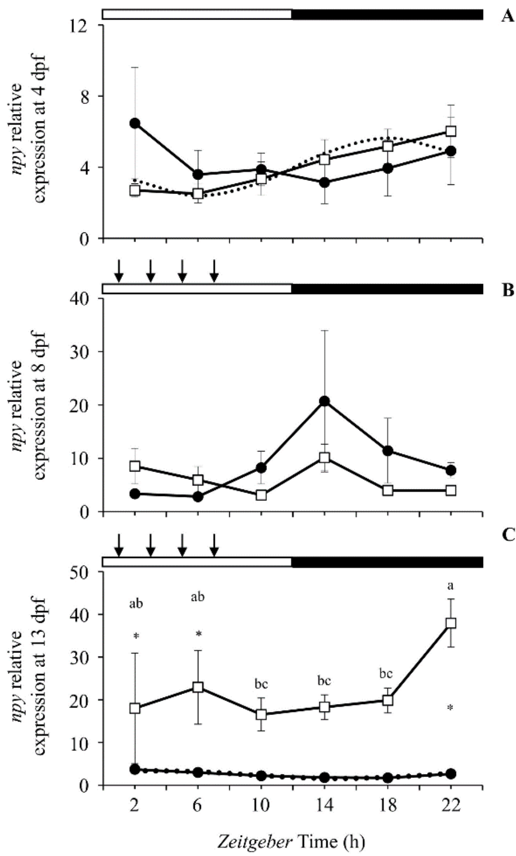


Fig. 8. Daily variations in the relative mRNA expression (fold change) of neu- ropeptide Y (npy) analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinu- soidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

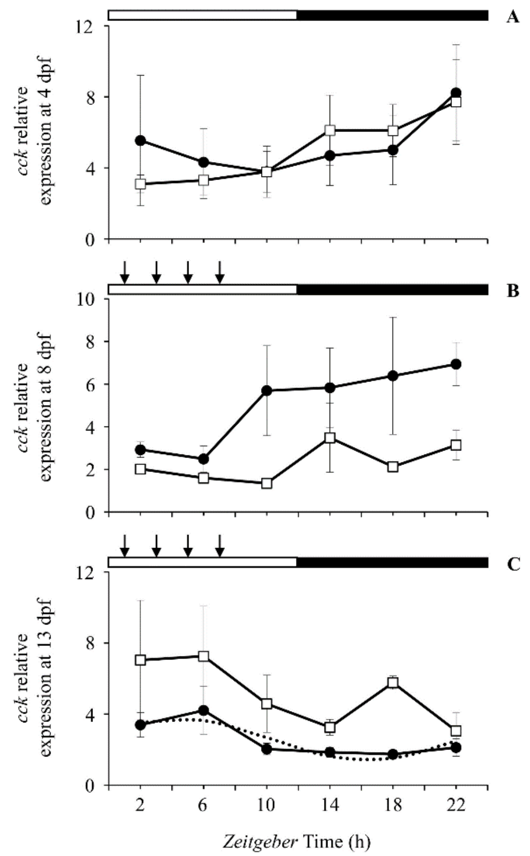


Fig. 9. Daily variations in the relative mRNA expression (fold change) of cho- lecystokinin (cck) analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinu- soidal rhythm whenever the Cosinor test was significant. The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

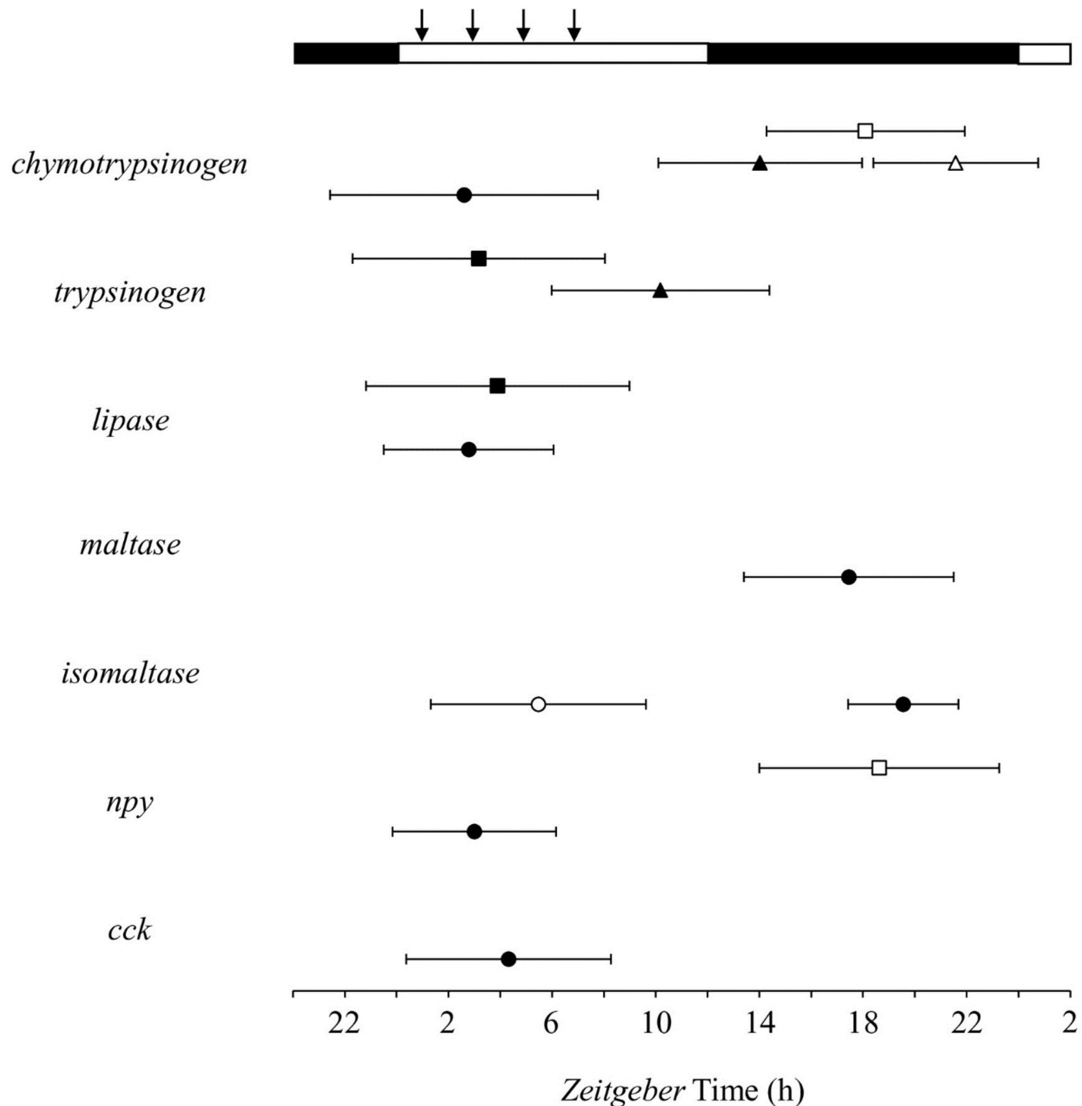
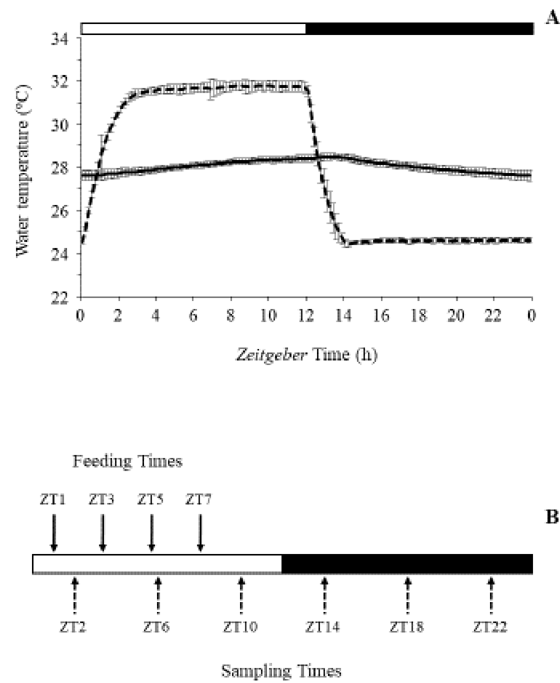
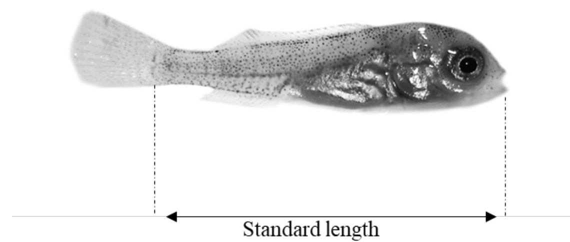


Fig. 10. Map of the acrophases of the digestive factors and hormones analyzed in tilapia larvae from 4, 8 and 13 days post fertilization (dpf) at a 12:12 LD cycle and by two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C or constant temperature (CTE) of 28 °C. The acrophases from the larvae reared in the TC group on 4, 8 and 13 dpf are represented by black squares, triangles and circles, respectively. The acrophases from the larvae reared in the CTE group on 4, 8 and 13 dpf are represented by white squares, triangles and circles, respectively. The acrophase is indicated only for the statistically significant rhythms (Cosinor, $p < .05$). The name of each represented gene is indicated on the left. The white and black bars above the graph represent the light and dark phases, respectively. The arrows above the graph indicate feeding times for 8 and 13 dpf groups. The time scale (x-axis) is expressed as Zeitgeber Time (ZT), in which ZT 0 h corresponds to light onset.



Supplementary Fig. 1. (A) Daily average water temperature (mean ± S.D.) throughout the experiments in the two temperature regimes herein tested: a thermocycle (TC) of 31 °C:25 °C (dashed line) or constant temperature (CTE) of 28 °C (continuous line). (B) Scheme of the feeding (continuous arrows) and sampling times (dashed arrows) used in the experiments. The white and black bars represent the light and dark phases, respectively. Time scale is expressed as Zeitgeber Time (ZT), in which ZT 0 h corresponds to light onset.



Supplementary Fig. 2. Standard tilapia larvae length measurement.

4. Discussion

In the present study, the existence of daily rhythms in the mRNA expression of several genes encoding different digestive enzymes and hormones were analyzed in tilapia larvae, as were the effects of temperature regime on the ontogeny of these rhythms. In general, rearing tilapia larvae under a thermocycle improved the occurrence of daily rhythms in these factors as most displayed significant rhythms on 13 dpf, which was not observed in the larvae maintained at constant temperature (Fig. 10). The acrophases (time of maximum value) of the factors involved in protein and lipids digestion, as well as hormones, were located in the morning, at around mealtime, on 13 dpf in the TC group (Fig. 10). The acrophases of the factors involved in carbohydrate digestion were located at night (Fig. 10). This greater number of significant rhythms occurred together with a higher growth as the TC larvae length was longer than that of the CTE group on 13 dpf.

When fish develop under cycling conditions, which are similar to the oscillating environmental conditions they experience in nature, they tend to perform better. In a previous study, tilapia juveniles presented higher growth rates under thermocycles than at constant temperatures, although this effect was lost as fish were becoming bigger and size dispersal was greater (Azaza et al., 2010). In addition, Senegalese sole and zebrafish (Danio rerio) present higher growth rates and a lower incidence of malformations when they are kept, during the early development, in thermocycles compared to constant temperatures (Blanco-Vives et al., 2010, Blanco-Vives et al., 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018). This agrees with the results obtained in the present study in tilapia growth with thermocycles. In those previous studies, however, the only analyzed molecular or physiological markers to explain the effects on development induced by thermocycles were some genes involved in sex differentiation as thermocycles also affect sex ratios (Blanco-Vives et al., 2010, Blanco-Vives et al., 2011; Villamizar et al., 2012). Therefore, in the present study, we focused on the analysis of factors involved in food intake control and digestion to investigate whether differences in growth may be explained by differences in these factors.

Regarding factors involved in food intake control, we analyzed npy and cck mRNA expression. In one hand, Npy is a peptidic hormone mainly produced in the brain (Volkoff, 2006). This hormone is the most potent orexigenic factor in vertebrates, including fish (Narnaware et al., 2000; Delgado et al., 2017). Besides its main effects as a stimulator of food consumption, Npy is a growth hormone (Gh) regulator (Peng and Peter, 1997). On the other

hand, Cck is also a peptidic hormone which is synthesized in the brain and gut (Volkoff et al., 2005). Cck is one of the most important regulators of digestion in fish and is largely responsible for the secretion of pancreatic enzymes (Koven et al., 2002; Volkoff et al., 2005; Zhang et al., 2018). Besides, Cck acts as a satiety (anorexigenic) signal in fish (Volkoff et al., 2005). In the present study, npy and cck presented rhythms with the highest values at around mealtime in the 13 dpf larvae. These rhythms were observed in larvae reared at TC but not at CTE. Thus one hypothesis that could explain the higher growth in the TC larvae is that the rhythms in these hormones, synchronized with mealtime, might have improved the digestive processes in these larvae compared to the CTE larvae.

Therefore, we analyzed the rhythms of expression of several enzymes involved in digestive processes. The existence of daily rhythms in digestive enzymes in fish was first described in adults (Vera et al., 2007; Montoya et al., 2010; López-Olmeda et al., 2012). In adult tilapia, daily rhythms have been reported in total protease activity, both acid and alkaline (Guerra-Santos et al., 2017). In recent years, the ontogeny of rhythms in digestive factors has been reported in the larvae of two marine species: gilthead sea bream and Senegalese sole (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016; Zeytin et al., 2016). In both species, most of the larval stages analyzed had already started the exogenous feeding and, under these conditions, daily rhythms in digestive enzymes correlated with feeding (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016). This is similar to the result herein found for the TC larvae on 13 dpf, where proteases (chymotrypsinogen and trypsinogen) and lipase presented the highest values around feeding time. The food digestion process depends, among other factors, on the presence and availability of adequate digestive enzymes throughout the gastrointestinal tract (Gisbert et al., 2013). The enzymes observed to display rhythms in tilapia larva are among the most important for protein (trypsin and chymotrypsin) and lipid digestion (lipase acting to release fatty acids and glycerol) (Almeida et al., 2018; Durigon et al., 2019). Taken together, the rhythms in both digestive enzymes and hormones might have increased the efficiency of physiological processes, leading to improved efficiency of food intake, digestion and growth in the TC larvae compared with the CTE group.

Moreover, maltase and isomaltase presented shifted acrophases and peaked at nighttime. These genes encode for enzymes that act in the final steps of the digestion of dietary carbohydrates (Tengjaroenkul et al., 2002). The peaks of maltase and isomaltase could have occurred later than other enzymes given the process phase in which they are involved. In addition, carbohydrate digestibility is generally poor in fish, although omnivorous fish like

tilapia have better digestion rates (Moon, 2001; Kamalam et al., 2017). Thus one possibility could be that carbohydrates remained for longer in the gut and, hence, the expression of these enzymes was delayed. On the other hand, gene expression does not necessarily correlate with the activity of the encoded enzyme (Yúfera et al., 2018), which could be the case for maltase and isomaltase and would explain why their rhythms are shifted compared to mealtime and to the other analyzed factors. Indeed, a lack of correlation between mRNA expression and the activity of the enzyme encoded should not be discarded for other genes analyzed in the present study. Thus, further research is required concerning enzymatic activity.

Previous studies have highlighted the importance of maintaining embryos/larvae under rearing conditions that are similar to the natural conditions that animals experience in the wild. This mainly involves using environmental cycles instead of constant conditions. For instance, it has been reported that LD cycles are required for correct circadian clock maturation in fish, and constant lighting conditions (either darkness or light) can delay or even suppress the appearance of daily rhythms (Ziv and Gothilf, 2006; Martín-Robles et al., 2013; Cuesta et al., 2014; Di Rosa et al., 2015). The temperature factor has been less studied than light. In zebrafish, thermocycles are able to synchronize the circadian rhythms of embryos/larvae during the first days of development in the absence of lighting cues, which indicates that thermocycles are a strong environmental signal for the ontogeny of the clock in fish (Lahiri et al., 2014). Likewise in tilapia in the present study, thermocycles enhanced the ontogeny of digestive circadian rhythms, which suggest an important role of thermocycles on fish circadian clock development, at least for freshwater species. In addition, it should be highlighted that tilapia in the wild is subjected to daily oscillations in temperature (Patterson and Wilson, 1995; Ndiwa et al., 2016). This species has evolved under these conditions, which might explain the impact of thermocycles on its physiology.

Finally, in the present study, thermocycles seemed to enhance the rhythmic expression of several genes, which in turn might have led to higher growth performance observed at 13 dpf. The mechanisms of action of thermocycles on the regulation of gene expression and how they can impact fish physiology remains unknown to date. Lahiri et al. (2005) suggested that, in the zebrafish, temperature cycles may act on the promoter of some genes, increasing or decreasing their expression through its action on an unknown enhancer element. In the case of clock genes and clock controlled genes, temperature would modify their phase and amplitude (Lahiri et al., 2005). In the present study, thermocycles led to a higher number of digestive factors displaying rhythmic variations, which might have occurred through their action at the

gene promoter level. Strengthening rhythmic factors might have led to the differences observed in growth, i.e. by tuning the timing of feeding and digestive processes. However, with the available data, the link between these rhythms and growth cannot be confirmed so that further research is required to test this hypothesis.

5. Conclusions

In summary, our research shows that applying daily thermocycles during early development may improve the ontogeny and maturation of the daily rhythms of digestive enzymes and feeding regulatory hormones in tilapia. Larvae reared under thermocycles presented a higher growth at the last stage analyzed (13 dpf), which might be due to higher digestion rates and better food efficiency. Therefore, the use of thermocycles instead of constant temperatures in tilapia larviculture is interesting as it might help to improve rearing protocols, although understanding the mechanisms responsible for this response would require further research.

6. Acknowledgments

The present research was partially funded by projects CHRONOLIPOFISH (RTI2018-100678-A-I00), BLUESOLE (AGL2017-82582-C3-3-R) and CRONOFISH (RED2018-102487-T), granted by the Spanish Ministry of Science, Innovation and Universities, cofounded with FEDER, to JFLO and FJSV. The research was also partially funded by projects from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico)401117/2014-6, 308684/2017-6 and 308547/2018-7, granted to RKL and PAPR, and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). JFLO was funded by a “Ramón y Cajal” research fellowship granted by the Spanish MINECO (RYC-2016-20959).

7. References

Abramoff M. D., Magalhaes P. J., Ram S. J. 2004. Image Processing with ImageJ. Biophotonics Intern. 11, 36-42.

Bennett, Wayne A.; Beitinger, Thomas L., 1997. Temperature tolerance of the sheepshead minnow, Cyprinodon variegatus. Copeia, 77-87.

Blanco-Vives, B., Vera, L. M., Ramos, J., Bayarri, M. J., Mañanós, E., de Moura Pereira, M., Machado Evangelista, M., Gisbert, E., Romagosa, E. 2019. Nile tilapia broodfish fed high-protein diets: Digestive enzymes in eggs and larvae. Aquac. Res., 50(8), 2181-2190.

Blanco-Vives, B., Vera, L.M., Ramos, J., Bayarri, M.J., Mañanós, E., Sánchez-Vázquez, F.J., 2011. Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in Solea senegalensis, kaup. J. Exp. Zoo. A Ecol. Genet. Physiol., 315(3), 162-169.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O., Sánchez-Vázquez, F.J., 2010. Effect of daily thermo-and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture, *306*(1-4), 137-145.

Blier, P. U., Pelletier, D., Dutil, J. D., 1997. Does aerobic capacity set a limit on fish growth rate. Reviews in Fish. Sci. 5(4), 323-340.

Brett, J.R., 1971. Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerkd*). Am. Zool., *11*(1), 99-113.

Champe, P.C., Harvey, R.A., Ferrier, D.R., 2006. Bioquímica ilustrada. 3ª. Edição Porto Alegre: Artmed Editora.

Costa, L.S., Rosa, P.V., Fortes-Silva, R., Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2016a. Daily rhythms of the expression of genes from the somatotropic axis: the influence on tilapia (Oreochromis niloticus) of feeding and growth hormone administration at different times. Comp. Bioch. and Phys C., 181, 27-34.

Costa, L.S., Serrano, I., Sánchez-Vázquez, F.J., & López-Olmeda, J.F., 2016b. Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions. J. Comp. Phys. B., 186(6), 775-785.

Cuesta, I.H., Lahiri, K., Lopez-Olmeda, J.F., Loosli, F., Foulkes, N.S., Vallone, D., 2014. Differential maturation of rhythmic clock gene expression during early development in medaka (*Oryzias latipes*). Chronobiol. Int., 31(4), 468-478.

DeCoursey, P.J., 2004. The behavioral ecology and evolution of biological timing systems. In J. C. Dunlap, J. J. Loros, & P. J. DeCoursey (Eds.), Chronobiology: Biological timekeeping (p. 27–65). Sinauer Associates.

Di Rosa, V., Frigato, E., López-Olmeda, J.F., Sanchez-Vazquez, F.J., Bertolucci, C., 2015. The light wavelength affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae. PloS one, 10(7).

Fernandes, A.F.A., Alvarenga, É. R., Oliveira, D.A.A., Aleixo, C.G., Prado, S.A., Luz, R.K., Turra, E.M., 2013. Production of oocytes of Nile tilapia (*Oreochromis niloticus*) for in vitro fertilization via hormonal treatments. Reprod. Domest. Anim., 48(6), 1049-1055.

Food, F. A. O. (2018). Agriculture Organization of the United Nations. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

Furné, M., Hidalgo, M.C., Lopez, A., Garcia-Gallego, M., MoraleS, A.E., Domezain, A., Domezaine, J., Sanz, A., 2005. Digestive enzyme activities in Adriatic sturgeon Acipenser naccarii and rainbow trout *Oncorhynchus mykiss*, A comparative study. Aquaculture 250, 391– 398.

Guerra-Santos, B., López-Olmeda, J.F., de Mattos, B.O., Baião, A. B., Pereira, D.S.P., Sánchez-Vázquez, F.J., Fortes-Silva, R., 2017. Synchronization to light and mealtime of daily rhythms of locomotor activity, plasma glucose and digestive enzymes in the Nile tilapia (*Oreochromis niloticus*). Comp. Biochem. Phys. A, 204, 40-47.

Jobling, M., 1995. Simple indices for the assessment of the influences of social environment on growth performance, exemplified by studies on Arctic charr. Aquac. Int. 3(1), 60-65.

Kamalam, B.S., Medale, F., Panserat, S., 2017. Utilisation of dietary carbohydrates in farmed fishes: new insights on influencing factors, biological limitations and future strategies. Aquaculture, 467, 3-27.

Koven, W., Rojas-García, C., Finn, R., Tandler, A., Rønnestad, I., 2002. Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupeaharengus*. Mar. Biol., 140(6), 1241-1247.

Lahiri, K., Froehlich, N., Heyd, A., Foulkes, N. S., Vallone, D., 2014. Developmental stage-specific regulation of the circadian clock by temperature in zebrafish. BioMed Res. Int., 2014.

Livak, K.J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.

López-Olmeda, J. F., López-García, I., Sánchez-Muros, M. J., Blanco-Vives, B., Aparicio, R., Sánchez-Vázquez, F. J., 2012. Daily rhythms of digestive physiology, metabolism and behaviour in the European eel (*Anguilla anguilla*). Aquac. Int. 20(6), 1085-1096.

López-Olmeda, J.F., 2017. Nonphotic entrainment in fish. Comp. Biochem. Phys. A., 203, 133-143.

López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2011. Thermal biology of zebrafish (Danio rerio). J. Therm. Biol., *36*(2), 91-104.

Martín-Robles, Á.J., Whitmore, D., Pendon, C., Munoz-Cueto, J.A., 2013. Differential effects of transient constant light-dark conditions on daily rhythms of Period and Clock transcripts during Senegalese sole metamorphosis. Chronobiol. Int., 30(5), 699-710.

Mata-Sotres, J.A., Moyano, F.J., Martínez-Rodríguez, G., Yúfera, M., 2016. Daily rhythms of digestive enzyme activity and gene expression in gilthead seabream (*Sparus aurata*) during ontogeny. Comp. Biochem. Phys. A. 197, 43-51.

Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J. (2010). Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (Sparus aurata). Aquaculture, 306(1-4), 315-321.

Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction?. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 129(2-3), 243-249.

Narnaware, Y.K., Peyon, P.P., Lin, X., Peter, R.E., 2000. Regulation of food intake by neuropeptide Y in goldfish. Am. J. Physiol. Regul. Integr. Comp. Physiol., 279(3), 1025-1034.

Navarro-Guillén, C., Moyano, F. J., Yúfera, M., 2015. Diel food intake and digestive enzyme production patterns in *Solea senegalensis* larvae. Aquaculture. 435, 33-42.

Ospina-Alvarez, N., Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS one. 3, 2837-2008.

Pankhurst, N.W; King, H.R., 2010. Temperatura e reprodução de salmonídeos: implicações para a aquicultura. J. Fish Biol. 76, 69-85.

Peng, C., Peter, R. E., 1997. Neuroendocrine regulation of growth hormone secretion and growth in fish. Zool. Stud., 36, 79-89.

Perez-Casanova, J. C., Murray, H. M., Gallant, J. W., Ross, N. W., Douglas, S. E., Johnson, S. C., 2006. Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture. 251(2-4), 377-401.

Podrabsky, J.E., Somero, G.N., 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. J. Exp. Biol. *207*(13), 2237-2254.

Qiang, J., He, J., Yang, H., Sun, Y.L., Tao, Y.F., Xu, P., Zhu, Z.X., 2017. Dietary lipid requirements of larval genetically improved farmed tilapia, *Oreochromis niloticus* (L.), and effects on growth performance, expression of digestive enzyme genes, and immune response. Aquac. Res. 48(6), 2827-2840.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Reviews in Aquaculture, 5, 59-98.

Sánchez-Vázquez, F.J., 2011. Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in Solea senegalensis, Kaup. J. Exp. Zool. A. Ecol. Genet. Physiol., *315*(3), 162-169.

Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2018. Environmental cycles and biological rhythms during early development. In Emerging Issues in Fish Larvae Research (pp. 37-50). Springer, Cham.

Schaefer, J., & Ryan, A., 2006. Developmental plasticity in the thermal tolerance of zebrafish Danio rerio J. Fish Biol. 69(3), 722-734.

Silva W.S, Costa L.S, López-Olmeda J.F, Costa N.C.S, Santos W.M, Ribeiro P.A.P, Luz R.K., 2019. Gene expression, enzyme activity and performance of Nile tilapia larvae fed with diets of different CP levels. Animal 3, 1376-1384.

Tengjaroenkul, B., Smith, B.J., Smith, S.A., Chatreewongsin, U., 2002. Ontogenic development of the intestinal enzymes of cultured Nile tilapia, Oreochromis niloticus L. Aquaculture, *211*(1-4), 241-251.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., Rozen, S. G., 2012. Primer3—new capabilities and interfaces. Nucleic acids res., *40*(15), 115-115.

Vera, L.M., De Pedro, N., Gomez-Milan, E., Delgado, M.J., Sanchez-Muros, M.J., Madrid, J.A., Sanchez-Vazquez, F.J., 2007. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiol. behave., 90(2-3), 518-524.

Villamizar, N., Blanco-Vives, B., Migaud, H., Davie, A., Carboni, S., Sanchez-Vazquez, F. J., 2011. Effects of light during early larval development of some aquacultured teleosts: a review. Aquaculture, 315(1-2), 86-94.

Villamizar, N., Ribas, L., Piferrer, F., Vera, L.M., Sánchez-Vázquez, F.J., 2012. Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. PLoS ONE. 7(12).

Volkoff, H., Canosa, L.F., Unniappan, S., Cerda-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. comp. endocrinol., 142(1-2), 3-19.

Yúfera, M., Moyano, F.J., Martínez-Rodríguez, G., 2018. The digestive function in developing fish larvae and fry. From molecular gene expression to enzymatic activity. In Emerging Issues in Fish Larvae Research (pp. 51-86). Springer, Cham.

Zhang, C., Rahimnejad, S., Wang, Y.R., Lu, K., Song, K., Wang, L., Mai, K., 2018. Substituting fish meal with soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*): Effects on growth, digestive enzymes activity, gut histology, and expression of gut inflammatory and transporter genes. Aquaculture, 483, 173-182.

Ziv, L., Gothilf, Y., 2006. Circadian time-keeping during early stages of development. Proc. Natl. Acad. Sci., 103(11), 4146-4151.

CAPÍTULO 2

Ontogenia dos ritmos diários de fatores metabólicos da tilápia do Nilo (*Oreochromis niloticus*) mantidas em diferentes temperaturas (termociclo e uma temperatura constante)

Ontogenia dos ritmos diários de fatores metabólicos da tilápia do Nilo (*Oreochromis niloticus*) mantidas em diferentes temperaturas (termociclo e uma temperatura constante)

Amanda Hastenreiter do Espírito Santo[1*], Gonzalo de Alba[2], Leandro Santos Costas[3], Francisco Javier Sánchez-Vázquez[2], José Fernando López-Olmeda[2], Ronald Kennedy Luz[1], Paula Adriane Perez Ribeiro[1]

[1]Departamento de Zootecnia, Universidade Federal de Minas Gerais, Escola de Veterinária, Laboratório de Aquacultura, Avenida Antônio Carlos, nº 6627, CEP 31270-901 Belo Horizonte, Minas Gerais, Brasil.

[2]Departamento de Fisiología, Universidad de Murcia, Facultad de Biología, Avda. Teniente Flomesta, nº 5, CEP 30003 Murcia, España.

[3]Departmento de Zootecnia, Universidade Federal de Viçosa, Av. P H Rolfs, s/nº - Campus Universitário, Viçosa, 36570-900, Minas Gerais, Brasil

*Autor correspondente, Email: amandahastenreiter@gmail.com

Resumo

A variação cíclica diária de temperatura, é um dos principais sincronizadores do ritmo circadiano, além disso a temperatura da água pode influenciar no desenvolvimento embrionário e larvário dos peixes, podendo afetar diretamente em suas enzimas metabólicas. Embora a aplicação de termociclos em larvas de peixes melhore o crescimento e sistema digestivo, nada se sabe sobre seus efeitos no metabolismo. O objetivo do presente estudo foi avaliar o efeito de diferentes regimes de temperatura, ciclagem *versus* constante, sobre os ritmos diários dos fatores metabólicos de larvas da tilápia do Nilo (*Oreochromis niloticus*). Para isso, ovos fertilizados foram divididos em dois grupos um em termociclo (TC) de 31 ° C: 25 ° C dia: noite e outro grupo em temperatura constante de 28 ° C (CTE). O fotoperíodo foi ajustado para o ciclo claro / escuro de 12:12 h. As amostras foram coletadas a cada 4 horas durante um ciclo de 24 horas nos dias 4, 8 e 13 pos fertilização. Os níveis de expressão de *alanina aminotransferase* (*alt*), *aspartato aminotransferase* (*ast*), *málica*, *glicose-6-fosfato deseidrogenase* (*g6pd*), *fosfofrutoquinase* (*pfk*) e *piruvato quinase* (*pk*) foram analisados por qPCR. Os resultados mostraram que em 13dpf, a maioria dos genes analisados (*alt*, *ast*, *málica*, *g6pd* e *pfk*) apresentaram ritmos diários em TC, mas não no grupo mantido em temperatura constante, com a maioria das acrofase durante o período de alimentação. Um aumento no metabolismo de nutrientes pode melhorar o aproveitamento do alimento podendo assim, aumentar o desempenho das larvas.

Palavras-Chave: expressão genica, fisiologia metabólica, relógio biológico, larvicultura

Abstract

The daily variations of temperature are one of the main synchronizers of the circadian rhythms. In addition, water temperature influences the embryonic and larval development of fish and directly affects their metabolic processes. The application of thermocycles to fish larvae has been reported to improve growth and the maturation of the digestive system, but their effects on metabolism are poorly understood. The aim of the present study was to evaluate the effect of two different temperature regimes, cycling versus constant, on the daily rhythms of metabolic factors of Nile tilapia (Oreochromis niloticus) larvae. For this purpose, fertilized eggs were divided into two groups: one reared in a 31°C:25°C day:night thermocycle (TC) and another group maintained in a constant 28°C temperature (CTE). The photoperiod was set to a 12:12 h light/dark cycle. Samples were collected every 4 hours during a 24-hour cycle on days 4, 8 and 13 post fertilization (dpf). The expression levels of alanine aminotransferase (alt), aspartate aminotransferase (ast), malic enzyme, glucose-6-phosphate dehydrogenase (g6pd), phosphofructokinase (pfk) and pyruvate kinase (pk) were analyzed by qPCR. Results showed that, in 13 dpf animals, most of the genes analyzed (alt, ast, malic, g6pd and pfk) showed daily rhythms in TC, but not in the group kept at constant temperature, with most acrophases detected during the feeding period. An increase in nutrient metabolism around feeding time can improve food utilization and thus increase larval performance. Therefore, the use of thermocycles is recommended for tilapia larviculture.

Keywords: gene expression, metabolic physiology, biological clock, larviculture

1. Introdução

Devido as suas ótimas características de produção, a tilápia *(Oreochromis niloticus)* é uma das espécies mais produzidas mundialmente (FAO, 2018). Uma dessas características é a tolerância a uma ampla gama de temperaturas (Omondi et al., 2014; Ndiwa et al., 2016), já que este animal provém de águas que variam entre temperadas a quentes. Outra vantagem na produção de tilápia é a utilização de dietas formuladas desde o início de sua alimentação exógena (Luz et al., 2012). As exigências nutricionais de larvas de tilápia foram bastante estudadas nos últimos anos, todavia, o aprofundamento sobre sua fisiologia digestiva e metabólica é necessário (De Moura Pereira et al., 2019).

A eficiência da conversão alimentar pode depender da disponibilidade de enzimas envolvidas nesse processo (Jobling 1995; Perez-Casanova et al., 2006), principalmente durante o desenvolvimento inicial (Blier et al., 1997). Sendo assim, pesquisas sobre a ontogênese dessas enzimas em peixes podem elucidar certos aspectos de sua fisiologia nutricional e ajudar a resolver os desafios nutricionais na larvicultura (Furné et al., 2005). Até o momento poucos estudos abordam a ontogênese de fatores digestivos durante a fase larval de peixes (Tengjaroenkul et al., 2002; Qiang et al., 2017; De Moura Pereira et al., 2019; Silva et al., 2019) e quando se trata dos fatores metabólicos, este número se reduz (Ma et al., 2021).

Devido às mudanças ambientais sazonais e diárias causadas por ciclos físicos, os animais evoluíram e desenvolveram padrões rítmicos conhecidos como relógio biológico (Kulczykowska et al., 2010). Os ritmos biológicos permitem que os organismos cronometrem seus processos fisiológicos para ocorrer em momentos específicos em que a eficácia é a maior (DeCoursey, 2004). No ambiente natural, os ciclos de luz e temperatura, juntamente com a alimentação, são um dos principais sincronizadores dos ritmos biológicos, especialmente em animais ectotérmicos como peixes (López-Olmeda, 2017). A presença ou ausência de radiação solar gera um termociclo: durante o dia a temperatura sobe (a termofase ou fase de maior temperatura), enquanto durante a noite a temperatura cai (a criofase ou fase de menor temperatura) (Villamizar et al., 2011; Blanco-Vives et al., 2010; López-Olmeda & Sánchez-Vázquez, 2011). Sabe-se que a temperatura pode afetar o desempenho e atividade enzimática (Moura & Lanna 2012; Bendhack et al., 2013), aproveitamento do alimento (Okamoto et al., 2006), locomoção (Bennett e Beitinger 1997), diferenciação sexual (Ospina-A´ & Francesc Piferre, 2008) reprodução (Nascimento et al, 2006; Pankhurst & King, 2010), sobrevivência dos peixes (Pankhurst & Munday 2011). Além disso, foi relatado que o uso de condições

cíclicas de temperaturas podem aumentar a sobrevivência, o crescimento e a expressão de enzimas digestivas, além de diminuir a incidência de má formação e modificam a razão sexual durante o período larval (Blanco-Vives et al., 2010, Blanco-Vives et al., 2011 ; Villamizar et al., 2012; Sánchez-Vázquez & López-Olmeda, 2018; Espirito Santo et al., 2020). A maioria dos peixes experimenta naturalmente termociclos, porém, pouco se sabe sobre os efeitos da variabilidade térmica na biologia dos peixes, principalmente no seu desenvolvimento inicial (Schaefer & Ryan, 2006; Villamizar et al., 2011).

Sendo assim, o objetivo deste estudo foi avaliar os efeitos de dois diferentes regimes de temperatura, um termociclo diário *versus* temperatura constante, aplicado nos primeiros dias de desenvolvimento da tilápia do Nilo, sobre os ritmos diários de diferentes parâmetros do metabolismo nutricional. Esses parâmetros envolveram a expressão de genes que codificam enzimas para o metabolismo de proteínas, lipídios e carboidrato.

2. Material e métodos

A presente pesquisa foi realizada no Departamento de Fisiologia da Universidade de Murcia (Espanha). Os peixes foram criados de acordo com a legislação espanhola sobre Bem-Estar Animal e Práticas Laboratoriais. Os protocolos experimentais foram realizados seguindo as Diretrizes da União Europeia (2010/63 / UE) e a legislação espanhola (RD 53/2013 e Lei 32/2007) para o uso de animais de laboratório. Além disso, os protocolos foram aprovados pelo Comitê Nacional e pelo Comitê de Ética e Bem-Estar Animal da Universidade de Murcia.

2.1 Animais e design experimental

Matrizes de tilápia do Nilo (*Oreochromis niloticus*) foram obtidas em uma fazenda de peixes local (Tilamur SA, Murcia, Espanha). Os animais foram mantidos em tanques de 300 litros conectados a sistema de recirculação, equipado com filtros biológicos e mecânicos. O fotoperíodo foi ajustado para o ciclo claro / escuro (LD) 12:12 h com as luzes acesas às 09:00 h. A temperatura da água foi mantida a 28 ± 0,5 ° C. As tilápias adultas foram alimentadas com dieta comercial (D-4 AlternaBasic 2P, Skretting, Espanha) com 36% de proteína bruta (PB) três vezes ao dia até a saciedade aparente.

Após a aclimatação, matrizes de tilápia foram induzidas com o hormônio gonadotrofina coriônica humana (hCG, Sigma Aldrich, St. Louis, EUA) conforme descrito por (Fernandes et al., 2013). As fêmeas foram injetadas intraperitonealmente (ip) com 1.500 UI / kg de peso

corporal e os machos com 500 UI / kg. A administração para as fêmeas foi dividida em duas injeções ip: a primeira consistiu em 500 UI / kg injetada no final da fase de luz e a segunda (1000 UI / kg) foi aplicada 12 h após a primeira dose (no início da fase de luz no dia seguinte). Os machos receberam uma única injeção ip ao mesmo tempo que a segunda dose para as fêmeas. Após a administração de hCG, os animais foram colocados juntos. Após 24 h, espermatozóides e óvulos foram coletados por extração e a fertilização *in vitro* foi realizada (Fernandes et al., 2013). Esse procedimento permitiu obter os ovos fertilizados, que foram usados nos experimentos.

Ovos fertilizados foram obtidos de cinco grupos diferentes de reprodutores de tilápia (3 fêmeas e 5–6 machos por grupo). Em seguida, os ovos no estágio 1 (Fujimura e Okada, 2007) foram agrupados e distribuídos em incubadoras para ovos de Cichlid (Alimar SA, Murcia, Espanha) (150-200 ovos por incubadora, foram utilizadas 6 incubadoras por grupo) em dois sistemas distintos, com diferentes regimes de temperatura: um sistema com um termociclo diário (TC) de 31° C: 25° C, e outro com uma temperatura constante (CTE) de 28° C. Ambos os sistemas eram de recirculação de água conectados a filtros mecânicos e biológicos. O fotoperíodo foi ajustado para 12:12 LD, com luzes acesas às 09:00 h. Por convenção, em estudos cronobiológicos o tempo de início da luz é definido como *Zeitgeber* (sincronizador) tempo 0 h (ZT0 h), a fim de padronizar os pontos temporais independente da hora local, facilitando a reprodutibilidade (Guerra-Santos et al., 2017). Portanto, o início da luz às 9:00 h corresponde a ZT 0 h e o deslocamento da luz até as 21:00 h corresponde a ZT 12 h. No sistema TC, a termofase (fase de alta temperatura) de 31° C coincidiu com a fase clara, enquanto a criofase (fase de baixa temperatura) de 25° C coincidiu com a fase escura (Figura suplementar 1A). A diferença de temperatura entre a termofase e a criofase foi selecionada de acordo com a literatura e os intervalos diários normais experimentados pela tilápia na natureza (Patterson &Wilson, 1995; Schaefer e Ryan, 2006 ; Villamizar et al., 2012; Ndiwa et al., 2016). A temperatura média da água no sistema TC ao longo do dia foi de 28° C. Assim, as larvas criadas neste sistema foram submetidas aos mesmos graus-dia que os animais criados no grupo CTE, a uma temperatura média da água de 28,0 ± 0,3° C (média ± DP). No sistema TC, a temperatura da água foi modificada por aquecedores de água (Askoll, Povolaro, Itália) e resfriadores (AquaMedic 1500, Titan GmbH, Bissendorf, Alemanha), controlados por temporizadores eletrônicos (Bachmann GmbH & Co, Stuttgart, Alemanha). A temperatura da água foi registrada continuamente durante todo o experimento por um gravador de dados subaquático (HOBO pendente, Onset Computer Corporation, Bourne, MA, EUA). As larvas foram criadas

em incubadoras até 7 dias pós a fertilização (dpf), quando foram transferidas para tanques de 9 litros, conectados ao mesmo sistema de temperatura. A densidade nos tanques foi de aproximadamente 10 larvas / l, e um total de dez tanques de 9 l foram usados nos experimentos (5 tanques por tratamento de temperatura). A alimentação exógena começou em 7 dpf. As larvas foram alimentadas com uma dieta semi-purificada (Tabela 1) contendo 42% PB e 4100 Kcal / kg, formulada conforme descrito por (Silva et al., 2019). As larvas foram alimentadas até saciedade aparente, 4 vezes ao dia na fase clara (ZT 1, ZT 3, ZT 5 e ZT 7 h) (Suppl. Fig. 1 B). Embriões / larvas de tilápia foram mantidos nas condições experimentais até 13 dpf.

Um total de 864 larvas de tilápia foram usadas nos experimentos para análises de qPCR. Em 4, 8 e 13 dpf, amostras inteiras de larvas foram coletadas a cada 4 h durante um ciclo de 24 h nos seguintes pontos de tempo: ZT 2, ZT 6, ZT 10, ZT 14, ZT 18 e ZT 22 h (Figura suplementar 1B). Foram utilizadas 6 repetições para cada ponto de tempo (ZT), cada uma delas constituída por um pool de larvas. Este pool variou conforme o tempo pós fertilização (dpf). Para 4 dpf foram utilizadas 5 larvas para cada pool; 4 larvas / pool em 8 dpf; e 3 larvas / pool em 13 dpf. As larvas foram armazenadas em tubos de 1,5 ml e imediatamente congeladas e armazenadas a −80 ° C até serem analisadas. As larvas coletadas nos dias 8 e 13 dpf foram alimentadas no dia da coleta.

2.2 Análise RT-PCR em tempo real

Os pools de larvas foram transferidos para tubos estéreis e homogeneizados em reagente Trizol (Ambion, Thermo Fisher Scientific, Waltham, EUA) seguindo as instruções do fabricante, utilizando um homogeneizador de tecido (TissueLyser LT, Qiagen, Hilden, Alemanha). O RNA foi dissolvido em água DEPC (Invitrogen, Carlsbad, EUA) e a concentração de RNA foi determinada por espectrometria (Nanodrop ND-1000, Thermo Fisher Scientific). O RNA total (1 µg) foi primeiro tratado com 1 U de DNase (Dnase I, Thermo Fisher), seguido pela retrotranscrição usando um kit comercial (qScript cDNA Synthesis Kit, Quantabio, Beverly, EUA). As reações de PCR quantitativo (qPCR) foram realizadas pelo SYBR Green PCR Master Mix (Applied Biosystems, Foster City, EUA). Todas as amostras foram executadas em duplicata e as reações qPCR foram realizadas em um volume final de 20 µl. As análises quantitativas de PCR foram realizadas em um termociclador leve (7500 RT-PCR system, Applied Biosystems) seguindo este protocolo: 15 min a 95 ° C, seguido de 40 ciclos de 15 s a 95 ° C e 1 min a 60 ° C. As curvas de fusão foram executadas após a amplificação para garantir que apenas uma espécie de DNA foi amplificada. Todas as

sequências de primer (Tabela 2) foram elaborados com o software Primer 3 plus (Untergasser et al., 2012). As eficiências de amplificação relativas de todos os genes foram analisadas por curvas de diluição de cDNA e foram consideradas semelhantes para todos os genes. As concentrações do primer determinadas por meio de uma curva de diluição. Os primers para *alanina aminotransferase* (*alt*), *aspartato aminotransferase* (*ast*), *fosfofrutoquinase* (*pfk*) e *βactina* foram adicionados numa concentração de 200 nM. Os primers para *piruvato quinase* (*pk*)*, enzima malíca* e *glicose-6-fosfato desidrogenase* (*g6pd*), adicionados numa concentração final de 400 nM. A expressão relativa, de todos os genes, foi calculada pelo método $2^{-\Delta\Delta Ct}$ (Livak e Schmittgen, 2001). O gene de referência *β-actina* foi selecionado após verificação de que seu coeficiente de variação (CV) era inferior a 5% e foi utilizado na primeira normalização. A amostra com o menor valor de expressão dentro de cada gene, tecido e grupo foi usada como referência para a segunda normalização.

O crescimento padrão das larvas de tilápia, em ambos os grupos de temperatura (TC e CTE), foi avaliado em estudo prévio, onde foram observadas diferenças significativas entre os grupos 13 dpf. Neste dia, as larvas do tratamento TC apresentaram valores de comprimento significativamente maiores (8,09 ± 0,07 mm, média ± SEM) em comparação as do tratamento CTE (7,77 ± 0,09 mm) (Espírito Santo et al., 2020).

2.3 Análise de dados

Todos os resultados são expressos como média ± SEM. O limite de significância foi estabelecido em $\alpha = 0{,}05$ para todos os testes. O software SPSS (v. 19.0, IBM, Armonk, EUA) foi usado para detectar quaisquer diferenças estatisticamente significativas entre os grupos (TC *versus* CTE) e pontos de amostragem (ZTs). A normalidade dos dados foi avaliada previamente pelo teste de Kolmogorov-Smirnov e a homogeneidade da variância também foi verificada pelo teste de Levene. Os dados foram submetidos a uma ANOVA de duas vias, seguida pelo teste *post hoc de* Duncan, para verificar diferenças estatisticamente significativas nas expressões gênicas entre os grupos (TC *versus* CTE) e pontos de amostragem (ZTs), e diferenças na duração entre os grupos e dias após a fertilização (4, 8 e 13 dpf).

A existência de ritmicidade significativa foi testada para todos os genes pela análise Cosinor, realizada com o software "EL TEMPS" (v.1.179, Prof. Díez-Noguera, Universidade de Barcelona, Espanha). A análise Cosinor é baseada na abordagem dos mínimos quadrados de dados de série temporal com uma função cosseno de um período conhecido do tipo Y = Mesor

+ Amplitude ∗ cos (2π (t-Acrofase) / Período). A análise de Cosinor também fornece a significância estatística do ritmo por um teste F da variância, contabilizado pela forma de onda *versus* uma linha reta de amplitude zero (hipótese nula).

3. Resultados

3.1 Expressão de *alanina aminotransferase*

A expressão relativa de *alt* apresentou ritmos diários significativos para as larvas de 4 dpf mantidas no grupo CTE (Cosinor, p = 0,026) e para as larvas de 13 dpf mantidas em TC (p = 0,001) (Fig. 1). As acrofases foram localizadas durante a fase clara, em ZT 3:36 h para 4dpf, em CTE, e em ZT 0:11 h para 13dpf, em TC (Tabela 2).

Além disso, a expressão de *alt* nas larvas com 4 dpf mostrou diferenças significativas dependendo do grupo (ANOVA de duas vias, p < 0,001), da hora do dia (p = 0,003) e da interação entre esses dois fatores (p = 0,007) (Fig. 1 A e Tabela Supl. 1). Neste mesmo estágio, a expressão de *alt* em CTE, em ZT 2, foi significativamente maior do que em TC no mesmo tempo, assim como os demais pontos em CTE.

3.2 Expressão de *aspartato aminotransferase*

O único ritmo diário significativo na expressão de *ast* foi detectado nas larvas de 13 dpf do grupo TC (Cosinor, p <0,01) (Fig. 2). A acrofase neste estágio ocorreu no início da fase clara ZT 0:37 h (Tabela 3).

Diferenças estatisticamente significativas foram encontradas na expressão de *ast* dependendo do fator grupo a 8 dpf (ANOVA de duas vias p <0,01) (Tabela Supl. 1) sendo maior no grupo TC. Já para os animais com 13 dpf (p = 0,01) também foram encontradas diferenças significativas dependendo do fator grupo, desta vez, sendo maior no grupo CTE em comparação com o TC (Tabela Supl. 1).

3.3 Expressão de *enzima málica*

Larvas com 4 e 8 dpf, em ambos os grupos TC e CTE, não apresentaram ritmos diários (Cosinor, p>0,05) na expressão relativa de *enzima málica* (Fig. 3). Já para as larvas com 13 dpf no grupo TC, encontrou-se ritmo diário significativo (Cosinor, p> 0,01), com a acrofase localizada na fase clara em ZT 5:54 h (Tabela 3).

Não foram observadas diferenças significativas (ANOVA de duas vias, p> 0,05), entre os grupos e tempo na expressão relativa de *enzima málica* em larvas com 4, 8 e 13 dpf, em ambos os grupos TC e CTE.

3.4 Expressão de *glicose-6-fosfato desidrogenase*

A expressão relativa de *g6fd* apresentou ritmos diários significativos para as larvas de 4 dpf, mantidas no grupo TC (Cosinor, p = 0,018), em 8 dpf para o grupo mantido em CTE (Cosinor, p=0,037) e para as larvas de 13 dpf, mantidas em TC (Cosinor, p = 0,038) (Fig. 4). As acrofases variaram entre os ritmos significativos detectados (Tabela 3). No grupo TC, a acrofase foi localizada durante a fase clara, ZT 1:36 h para larvas com 4dpf e ZT 2:37 h para larvas com 13dpf. Já para as larvas com 8dpf mantidas em CTE, a acrofase foi em ZT 22:05 h, no fim da fase escura.

Além disso, a expressão de *g6pd* nas larvas de 4 dpf exibiu diferenças significativas dependendo do grupo (ANOVA de duas vias, p> 0,01), da hora o dia (p = 0,013), e da interação entre estes dois fatores (p> 0,01) (Fig. 4A e Tabela suplementar 1). Neste estágio, a expressão de *g6pd* em TC, em ZT 2 h e ZT 6h foi significativamente maior do que CTE (Fig. 4). Já para os animais em 13 dpf, diferenças estatisticamente significativas foram encontradas dependendo do fator de grupo (p = 0,043) e a expressão geral de *g6pd* foi maior no grupo CTE em comparação com TC (Tabela suplementar 1).

3.5 Expressão *de fosfofrutoquinase*

A expressão relativa de *pfk* apresentou ritmos diários significativos para larvas de 4dpf mantidas no grupo TC (Cosinor, p = 0,044), em 8dpf mantidas em TC (Cosinor, p = 0,007), e para larvas de 13dpf mantidas em TC (Cosinor, p = 0,002) (Fig. 5). As acrofase variaram entre os ritmos significativos (Tabela 3), iniciando na fase escura em ZT 19:14 h para larvas com 4dpf, sendo arrastada para ZT 0:42 h, no início da fase clara, em larvas com 8dpf e, novamente ajustada para ZT 2:10 h em 13dpf.

Não foram observadas diferenças significativas (ANOVA de duas vias, p> 0,05), entre os grupos e tempos na expressão relativa de *pfk* em larvas com 4, 8 e 13 dpf (Tabela suplementar1).

3.6 *Expressão de piruvato quinase*

Ritmos diários significativos de *pk* foram encontrados a 8dpf em TC (Cosinor, p = 0,0013) (Fig. 6). Esse ritmo apresentou acrofase no final da fase escura em ZT 22:05 h (Tabela 3).

As larvas de 13dpf apresentaram diferenças significativas na expressão de *pk* dependendo do grupo (ANOVA de duas vias, p = 0,001) e na interação entre os fatores de grupo e tempo (p = 0,008) (Fig. 6C e Tabela suplementar 1). A expressão de *pk* no grupo CTE apresentou variações significativas entre os pontos de tempo, com maior expressão no ZT 14, ZT 18 e ZT 22 (Fig. 6 C e Tabela suplementar 1).

Tabela 1. Formulação da dieta experimental.

Ingredientes	Inclusão (%)
Caseína	41
Óleo de peixe	9
Óleo de soja	3,55
Inerte	16,5
Fosfato bicálcico	1,00
Sal (NaCl)	0,05
Premix de mineral/vitamina	1,00
Gelatina	8
Dextrina	19,9
Proteína Bruta (%)	42,44
Energia Bruta (Kcal/Kg)	4087,13

Tabela 2. Sequência de primers utilizada para análise de qPCR quantitativo.

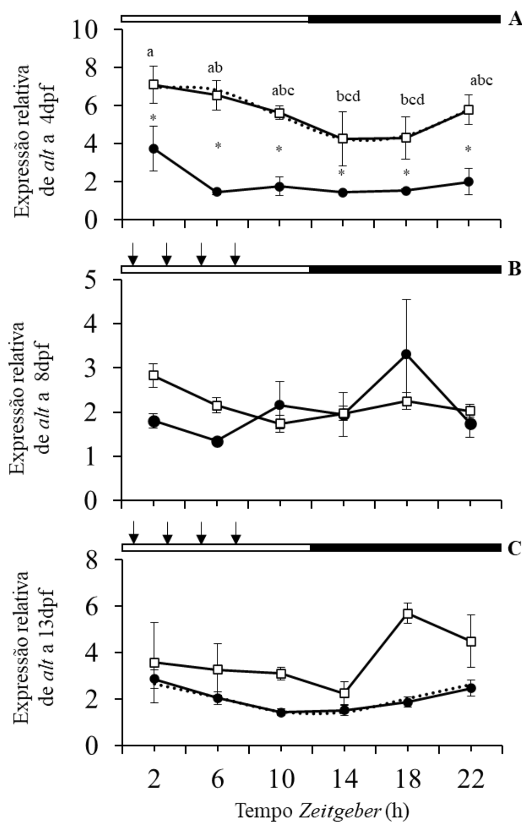
Gene	F/R	Sequência (5′-3′)	Ensembl/GenBank Número de acesso
alt	F	GGCAGCTTTATCAAGGAACG	XM_005476466.4
	R	TGGTTATGCGAGGAAAGGAG	
ast	F	AACATTGTGCTGTCGCAGTC	XM_003442255.5
	R	TCCTCTGCATCTTTGCACAC	
málica	F	TGTCCTTCAAGATCGCAGTG	XM_003452960.5
	R	ATCACACGTCCTGGACCTTC	
g6pd	F	GTCACCTCAACCGGGAAGTA	XM_013275693.3
	R	TGGCTGAGGACACCTCTCTT	
pfk	F	TTTGTGCATGAGGGTTACCA	XM_003441476.5
	R	CACCTCCAATCACACACAGG	
pk	F	TCCTGCAGACACAGATCCAG	ENSONIT00000010593.1
	R	TGATCATTTCCTTGGCCTTC	
βactina	F	TGGTGGGTATGGGTCAGAAAG	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA	

Tabela 3. Acrofase dos genes relacionados ao metabolismo de tilápia do Nilo submetida a um termociclo (31ºC: 25ºC) e uma temperatura constante (28 ºC).

Genes	dpf	Acrofase (ZT h)	
		TC	CTE
alt	4	-	3:36± 4:14*
	8	-	-
	13	0:11± 2:15**	-
ast	4	-	-
	8	-	-
	13	0:37± 2:08**	-
málica	4	-	-
	8	-	-
	13	5:54±3:11*	-
g6pd	4	1:36± 3:35*	-
	8	-	22:15± 4:39*
	13	2:37 ± 4:43*	-
pfk	4	19:14± 5:04*	-
	8	0:42± 3:21**	-
	13	2:10± 2:48*	-
pk	4	-	-
	8	22:05± 2:39**	-
	13	-	-

Cosinor*p<0,05**p<0,01.

A acrofase não está indicada para genes não significativos (p >0,05).



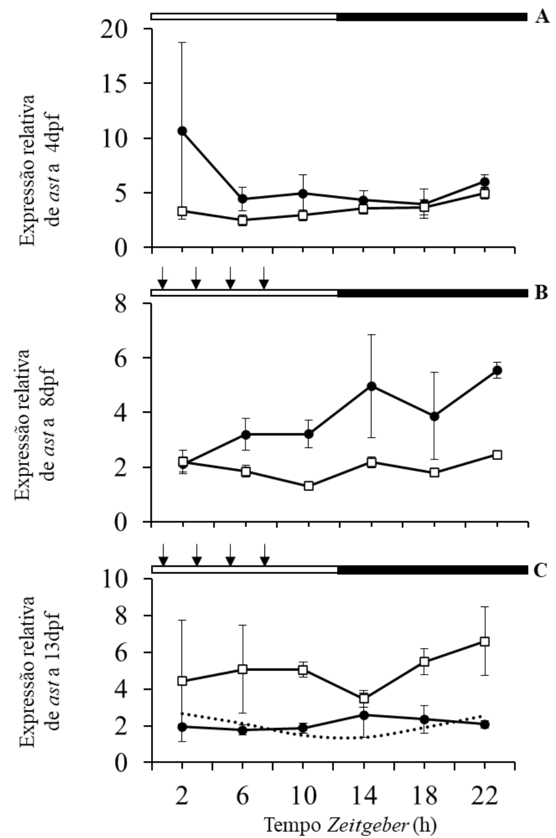


Figura 1. Variações diárias na expressão relativa do mRNA (mudança de *enovelamento*) de alanina *aminotransferase* (*alt)* em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa como*Zeitgeber* Time (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; *n* = 6 repetições (pools de larvas) por ponto.

Figura 2. Variações diárias na expressão relativa do mRNA (mudança de *enovelamento*) de aspartato aminotransferase (*ast)* em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa como *Zeitgeber* Time (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; *n* = 6 repetições (pools de larvas) por ponto.

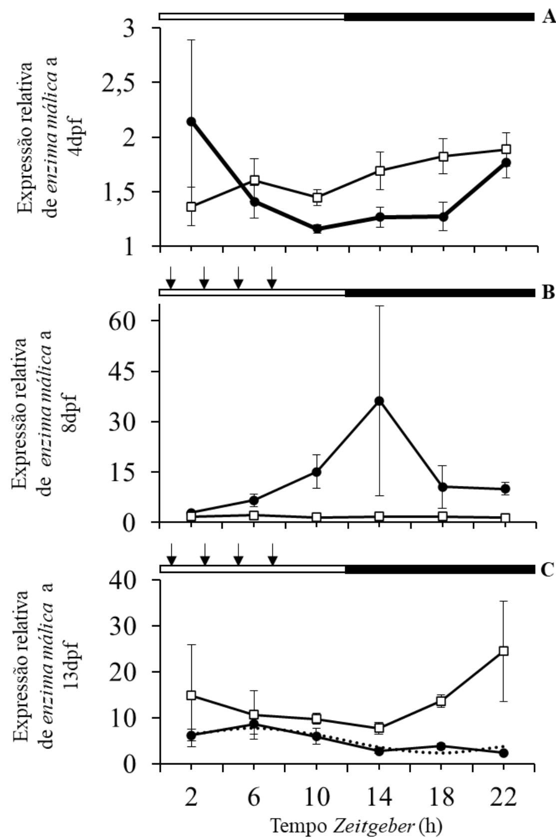


Figura 3. Variações diárias na expressão relativa do mRNA (mudança de *enovelamento*) de enzima málica em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa como tempo *Zeitgeber* (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; *n* = 6 repetições (pools de larvas) por ponto.

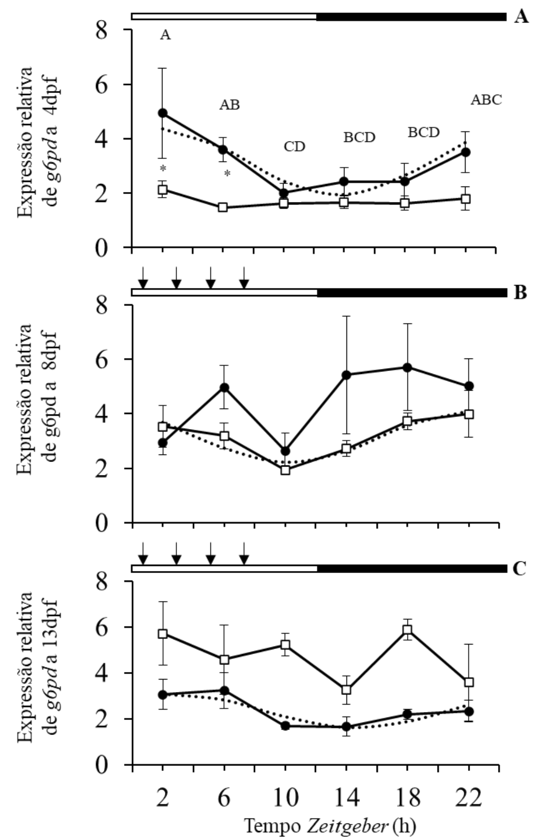


Figura 4. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de glicose-6-fosfato desidrogenase (g6pd) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa como tempo Zeitgeber (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; n = 6 repetições (pools de larvas) por ponto.

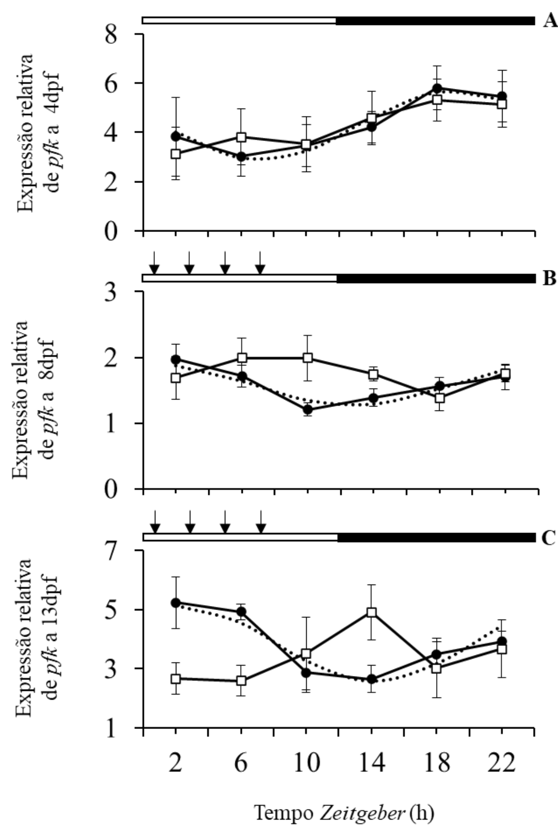


Figura 5. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de fosfofrutoquinase (pfk) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa comoZeitgeber Time (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; n = 6 repetições (pools de larvas) por ponto.

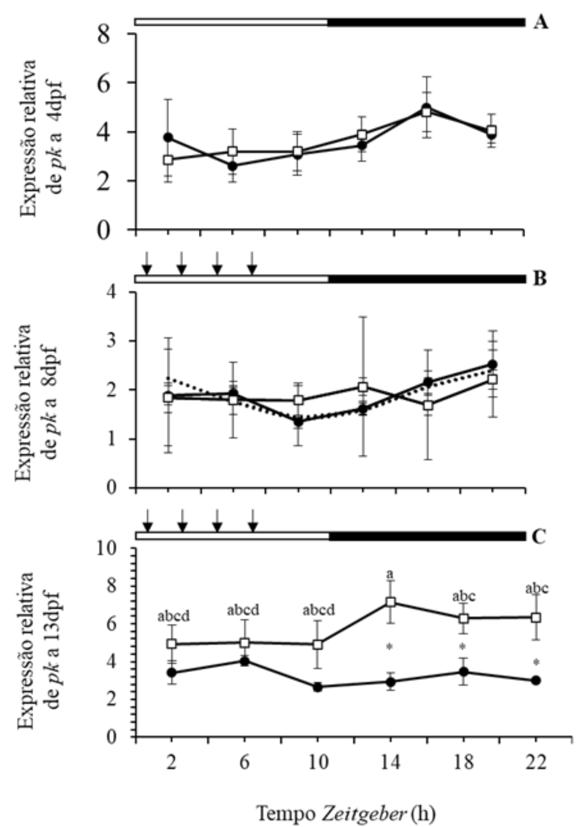


Figura 6. Variações diárias na expressão relativa do mRNA (mudança de enovelamento) de piruvato quinase (pk) em larvas de tilápia em 4 (A), 8 (B) e 13 (C) dias pós fertilização (dpf) em um ciclo de LD 12:12. As larvas foram submetidas a dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C (representado por círculos pretos, ●) ou temperatura constante (CTE) de 28 ° C (representada por quadrados brancos, □). A linha tracejada sinusoidal representa o ajuste a um ritmo sinusoidal sempre que o teste de Cosinor foi significativo. Letras maiúsculas e minúsculas diferentes indicam diferenças estatisticamente significativas entre os pontos de tempo dentro dos grupos TC ou CTE, respectivamente, no mesmo gráfico (ANOVA bidirecional). Os asteriscos indicam diferenças significativas entre os grupos no mesmo ponto de tempo (ANOVA de dois fatores). As barras brancas e pretas acima de cada gráfico representam a fase clara e a fase escura, respectivamente. As setas acima dos gráficos de 8 e 13 dpf indicam os tempos de alimentação. A escala de tempo (eixo x) é expressa comoZeitgeber Time (ZT), onde ZT 0 h corresponde ao início da luz. Os dados são representados como média ± SEM; n = 6 repetições (pools de larvas) por ponto.

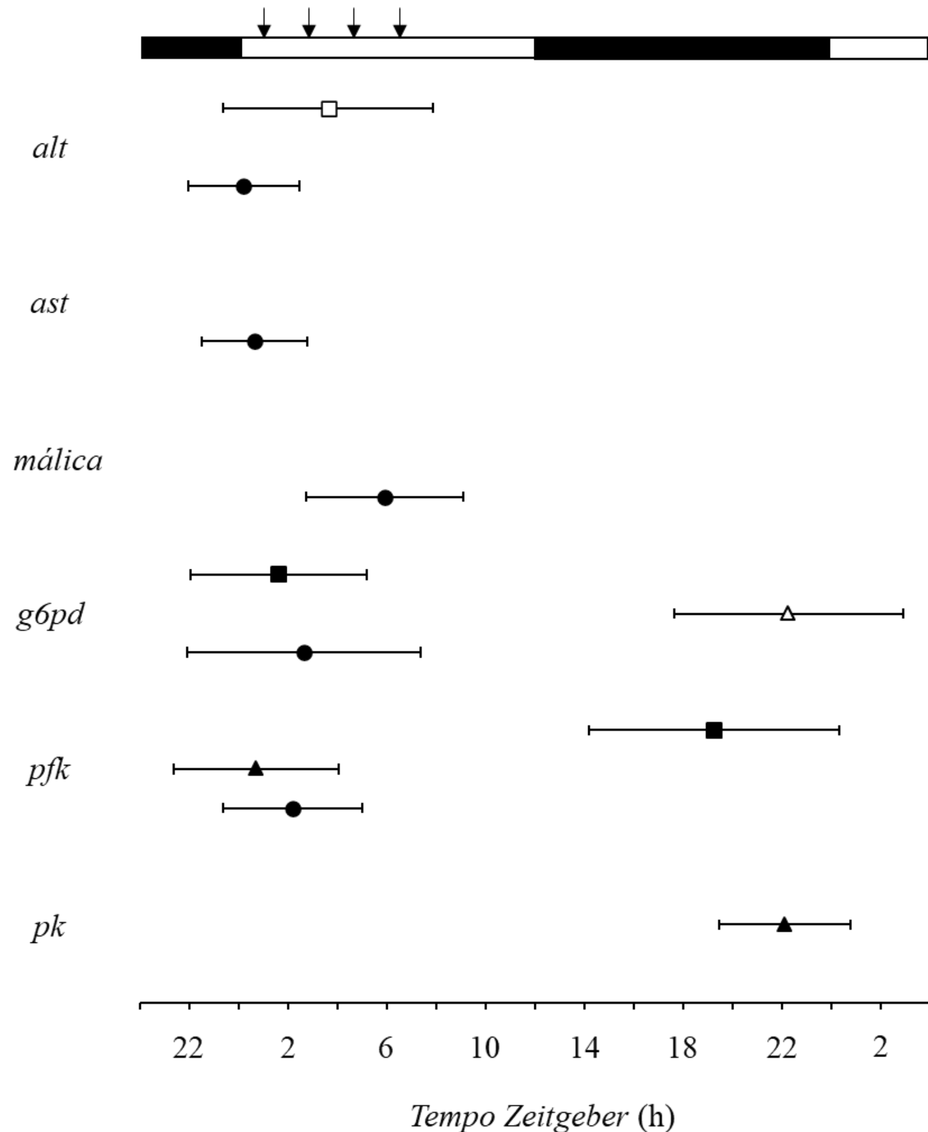


Fig. 7. Mapa das acrofases dos fatores digestivos e hormônios analisados em larvas de tilápia de 4, 8 e 13 dias pós-fertilização (dpf) em um ciclo LD 12:12 e por dois regimes de temperatura diferentes: um termociclo (TC) de 31 ° C: 25 ° C ou temperatura constante (CTE) de 28 ° C. As acrofases das larvas criadas no grupo TC em 4, 8 e 13 dpf são representadas por quadrados pretos, triângulos e círculos, respectivamente. As acrofases das larvas criadas no grupo CTE em 4, 8 e 13 dpf são representadas por quadrados brancos, triângulos e círculos, respectivamente. A acrofase é indicada apenas para os ritmos estatisticamente significativos (Cosinor, $p < 0,05$). O nome de cada gene representado é indicado à esquerda. As barras brancas e pretas acima do gráfico representam as fases de claro e escuro, respectivamente. As setas acima do gráfico indicam os tempos de alimentação para grupos de 8 e 13 dpf. A escala de tempo (eixo x) é expressa como Tempo Zeitgeber (ZT), em que ZT 0 h corresponde ao início da luz.

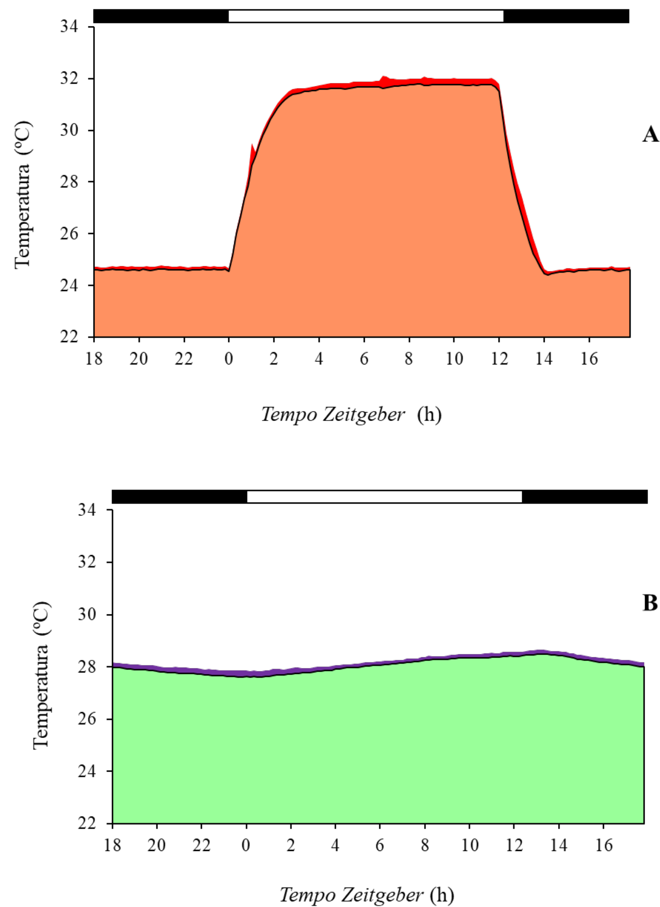


Figura suplementar 1. Temperatura média diária da água (média ± DP) ao longo dos experimentos nos dois regimes de temperatura aqui testados: um termociclo (TC) de 31 ° C: 25 ° C (A) ou temperatura constante CTE de 28 ° (B).

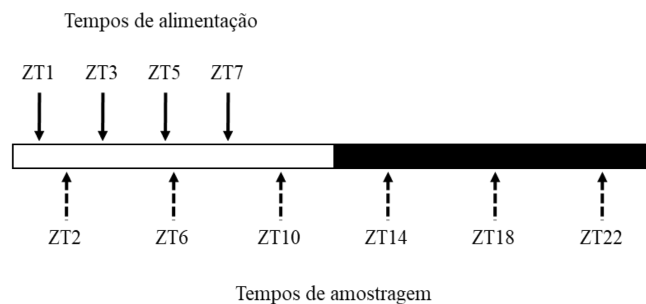


Figura suplementar 2. Esquema de alimentação (setas contínuas) e tempos de amostragem (setas tracejadas) usados nos experimentos. As barras brancas e pretas representam as fases clara e escura, respectivamente. A escala de tempo é expressa como Tempo Zeitgeber (ZT), em que ZT 0 h corresponde ao início da luz.

Tabela suplementar 1. Valores de P obtidos nas ANOVAs de dois fatores. Os valores de p são reportados para o grupo (G), tempo (T) e a interação desses dois fatores (GxT). A cor cinza destaca os resultados significativos (p <0,05).

Gene	4 dpf			8 dpf			13 dpf		
	G	T	GxT	G	T	GxT	G	T	GxT
alt	0.001	0.031	0.001	0.716	0.408	0.423	0.042	0.099	0.161
ast	0.310	0.359	0.361	0.008	0.639	0.220	0.01	0.765	0.326
málica	0.323	0.279	0.151	0.054	0.631	0.466	0.089	0.685	0.576
g6pd	0.001	0.013	0.001	0.058	0.267	0.296	0.043	0.377	0.387
pfk	0.936	0.148	0.569	0.183	0.499	0.222	0.311	0.875	0.146
pk	0.945	0.286	0.741	0.932	0.380	0.820	0.001	0.720	0.008

4. Discussão

Foram analisados neste estudo a existência de ritmos diários na expressão do mRNA de alguns genes que codificam enzimas metabólicas em larvas de tilápia, assim como os efeitos do regime de temperatura na ontogenia desses ritmos. No geral, foi observado que as larvas de tilápias mantidas em TC, apresentaram ritmos significativos para todas as enzimas metabólicas avaliadas, o que não foi observado para as larvas mantidas em CTE, onde os ritmos foram significativos apenas para as enzimas alt e g6pd (Fig. 7). Todas as acrofases (tempo de valor máximo), localizaram-se pela manhã, por volta das três primeiras alimentações a 13 dpf no grupo TC (Fig. 7).

Os peixes tendem a se desenvolver melhor quando estão em condições semelhantes às condições ambientais. A ritmicidade dos fatores que influenciam o ambiente natural faz com que os animais, se preparem para lidar com esses eventos cíclicos (Volpato & Trajano, 2006). Como foi observado no estudo prévio a este, as larvas de tilápia mantidas em TC com 13 dpf tiveram um crescimento significativamente maior que as larvas com a mesma idade mantidas em CTE (Espríto Santo et al., 2020). Em estudos anteriores, juvenis de tilápia, linguado (*Solea solea*) e peixe-zebra (*Danio rerio*), apresentaram maior taxa de crescimento em TC do que em temperaturas contantes (Azaza et al., 2010; Blanco-Vives et al., 2010; Blanco-Vives et al., 2011; Villamizar et al., 2012; Sánchez-Vázquez & López-Olmeda, 2018). Porém os únicos marcadores moleculares ou fisiológicos analisados para explicar esses

efeitos foram alguns genes envolvidos na diferenciação sexual (Blanco-Vives et al., 2010, Blanco-Vives et al., 2011 ; Villamizar et al., 2012).

Além dos fatores nutricionais, que já são bastante conhecidos, outros fatores como, genéticos e ambientais, devem ser levados em consideração quando se busca um melhor processo de desenvolvimento larval. A ingestão de alimento, taxa metabólica e o desenvolvimento dos organismos ectotérmicos são fortemente dependentes da temperatura (López-Olmeda & Sánchez-Vázquez, 2011). Alguns trabalhos vem demonstrando a existência de ritmos diários nas enzimas digestivas em peixes, adultos e larvas (Vera et al., 2007 ; Montoya et al., 2010 ; López-Olmeda et al., 2012; Navarro-Guillén et al., 2015 ; Mata-Sotres et al., 2016; Zeytin et al. , 2016; Guerra-Santos et al., 2017; Espírito Santo et al., 2020). No entanto, são escassos os trabalhos que avaliam a existência de ritmos nas enzimas metabólicas dos peixes.

As enzimas metabólicas refletem, entre outros fatores, a absorção e o metabolismo dos nutrientes, por isso, no presente estudo, foram avaliados os ritmos de expressão genica das enzimas envolvidas no processo metabólico de proteína (*alt* e *ast*), lipídios (*malica* e *g6pd*) e carboidrato (*pfk* e *pk*). A transaminação é o primeiro passo no processo de desaminação proteica, onde ocorre a transferência da amônia do grupo α-amino para um α-cetoácido. Existem diferentes enzimas transaminases, ou aminotransferases, porém as mais estudadas em peixes são ALT e AST. Sendo assim, a presença das enzimas ALT e AST indicam o processo de catabolismo de aminoácidos. As enzimas málicas e G6PD, participam dos processos lipogênicos fornecendo energia para os processos bioquímicos. Já as enzimas PFK e PK são enzimas- chave da via glicolítica. Um recente estudo, relata que o uso de variações cíclicas no ambiente, juntamente com a alimentação exógena, proporcionaram um aumento na atividade das enzimas ALT e AST para larvas de baiacu (*Tetraodontidae*) (Ma et al., 2021). Esta pesquisa nos mostra, que os animais com 13 dpf em TC apresentam ritmos diarios para *alt*, *ast*, *malica*, *g6pd* e *pfk* e não para as larvas mantidas em CT. Podemos sugerir que, os animais mantidos em condições cíclicas de temperatura com 13dpf, aumentam a eficiência dos seus processos fisiológicos, aumentando assim, a metabolização dos nutrientes.

É importante ressaltar que as larvas com 13dpf já haviam iniciado a alimentação exógena. As enzimas *alt* e *ast* apresentaram acrofase em ZT 0:11 e ZT 0:37 h (respectivamente), pouco depois de iniciar o período de luz e antes da primeira alimentação (ZT 1 h). Para as demais enzimas, *malica*, *g6pd*, *pfk*, a acrofase aconteceu entre a primeira e última alimentação

(ZT 1h – ZT 7 h). Com o passar dos anos, os peixes evoluíram e desenvolveram mecanismos para antecipar sua alimentação, desde que oferecida de forma programada e fixa (López-Olmeda & Sánchez-Vázquez, 2011; López-Olmeda et al., 2012). Fisiologicamente, os peixes são capazes de melhorar sua ingestão e utilização de nutrientes quando se antecipam a uma refeição (Comperatore & Stephan, 1987; Sánchez-Vázquez & Madrid, 2001). Alguns trabalhos vêm demonstrando, que a antecipação ao alimento aumenta níveis plasmáticos de hormônios importantes para a ingestão e digestão de alimentos em peixes, além de aumentar a atividade e expressão enzimática de enzimas envolvidas na digestão alimentar (Vera et al., 2007; Montoya et al.,2010; López-Olmeda et al., 2012; Prieto-Guevara et al., 2015; Espírito Santo et al., 2020). Sendo assim, podemos sugerir que as larvas de tilápias mantidas em TC com 13 dpf estavam se antecipando a alimentação, aumentando o metabolismo dos nutrientes (Montoya et al., 2010) e consequentemente otimizando o seu uso (Sánchez-Vázquez & Madrid, 2001) podenso melhorar o crescimento do animal (Prieto-Guevara et al., 2015; Espírito Santo et al., 2020).

A temperatura é um fator abiótico importante já que pode impactar diretamente os aspectos fisiológicos (Koumoundouros et al., 2002) e bioquímicos, podendo aumentar ou diminuir o metabolismo dos peixes (Fauconneau, 1985; Blier et al., 1997). A tilápia, no seu ambiente natural, sofre oscilações diárias de temperatura (termociclos) (Omondi et al., 2014; Ndiwa et al., 2016), e durante seu período larval são mais susceptíveis a esses efeitos (Rombough et al., 1997). O sistema circadiano de peixes é desenvolvido durante as primeiras fases do período larval (Kazimi & Cahill, 1999) e a correta maturação do relógio circadiano é influenciada pelo ciclos ambientais. Já, o contrário, ou seja, o uso de condições constantes podem atrasar ou suprimir o aparecimento de ritmos diários (Ziv & Gothilf, 2006; Martín-Robles et al., 2013; Cuesta et al., 2014; Di Rosa et al., 2015). Já foi demonstrado que o termociclo é capaz de sincronizar os ritmos circadianos, sendo assim um forte sinal ambiental para a ontogenia do relógio em peixes (Lahiri et al. , 2014). Alguns trabalhos demonstraram que a utilização de termociclos aumentam a secreção enzimática e melhoram o desempenho dos animais e o seu crescimento (Blanco-Vives et al., 2010; Villamizar et al., 2012). Além disso, um estudo prévio a esse, nos mostra que o uso de termociclos durante o período larval, aumenta a expressão rítmica de vários genes envolvidos na ingestão e digestão de alimentos, o que por sua vez levou a um maior crescimento das larvas de tilápia (Espírito santo et al., 2020). Portanto, pode-se sugerir com o presente estudo, que o uso de termociclo para larvas de tilápia aumentam a ontogenia dos ritmos circadianos das enzimas metabólicas, após 13 dpf. Aumentando a metabolização e o aproveitamento de nutrientes, as larvas poderiam aumentar

seu desempenho e crescimento. Ainda não se sabe quais os mecanismos de ação dos termociclo na regulação e expressão genica, de modo que mais pesquisas sobre esse tema devem ser desenvolvidas.

5. Conclusões

Nossa pesquisa mostra que larvas de tilápia, criadas sob termociclos diários, aumentam a expressão rítmica de genes envolvidos no metabolismo alimentar após 13dpf. Portanto o uso de termociclos em vez de temperaturas constantes na larvicultura de tilápia é recomendado, já que proporcionam maiores benefícios.

6. Agradecimentos

A presente pesquisa foi financiada pelos Projetos “CHRONOLIPOFISH” (RTI2018-100678-A-I00), concedidos pelo Ministério de Ciência, Inovação e Universidades da Espanha (MINECO), co-fundado com a FEDER, ao JFLO e ao CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq 401117 / 2014-6 e PQ CNPq-305048/ 2015-5), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). O JFLO foi financiado por uma bolsa de pesquisa “Ramón y Cajal”, concedida pelo espanhol MINECO (RYC-2016-20959).

7. Referencias

Azaza, M.S., Legendre, M., Kraiem, M.M., Baras, E., 2010. Size-dependent effects of daily thermal fluctuations on the growth and size heterogeneity of Nile tilapia Oreochromis niloticus. J. Fish Biol. 76, 669–683.

BENDHACK, F., PECZEK, V., GONÇALVES, R., & BALDAN, A. P. (2013). Desempenho do robalo-peva em diferentes temperaturas de cultivo. Pesquisa Agropecuária Brasileira, 48(8), 1128-1131.

BENNETT, WAYNE A.; BEITINGER, THOMAS L. Temperature tolerance of the sheepshead minnow, Cyprinodon variegatus. Copeia, p. 77-87, 1997.

BLANCO-VIVES, B., VILLAMIZAR, N., RAMOS, J., BAYARRI, M. J., CHEREGUINI, O., & SÁNCHEZ-VÁZQUEZ, F. J. (2010). Effect of daily thermo-and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture, 306(1-4), 137-145

Blanco-Vives, B., Vera, L.M., Ramos, J., Bayarri, M.J., Mañanós, E., Sánchez-Vázquez, F.J., 2011. Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in Solea senegalensis, kaup. J. Exp. Zool. A 315, 162–169. Blier, P.U., Pelletier, D., Dutil, J.D., 1997. Does aerobic capacity set a limit on fish growth rate. Rev. Fish. Sci. 5, 323–340.

COMPERATORE, C. A., STEPHAN, F. K. (1987). Entrainment of duodenal activity to periodic feeding. Journal of biological rhythms, 2(3), 227-242. Cuesta, I.H., Lahiri, K., López-Olmeda, J.F., Loosli, F., Foulkes, N.S., Vallone, D., 2014. Differential maturation of rhythmic clock gene expression during early development in medaka (Oryzias latipes). Chronobiol. Int. 31, 468–478.

Di Rosa, V., Frigato, E., López-Olmeda, J.F., Sanchez-Vazquez, F.J., Bertolucci, C., 2015. The light wavelength affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae. PLoS One 10 (7), e0132235.

De Moura Pereira, M., Machado, E.M., Gisbert, E.R.E., 2019. Nile tilapia broodfish fed high-protein diets: digestive enzymes in eggs and larvae. Aquac. Res. 50, 2181–2190.

DeCoursey, P.J., 2004. The behavioral ecology and evolution of biological timing systems. In: Dunlap, J.C., Loros, J.J., DeCoursey, P.J. (Eds.), Chronobiology: Biological Timekeeping. Sinauer Associates, Sunderland, pp. 27–65.

Espírito Santo, A. H., de Alba, G., da Silva Reis, Y., Costa, L. S., Sánchez-Vázquez, F. J., Luz, R. K., ... & López-Olmeda, J. F. (2020). Effects of temperature regime on growth and daily rhythms of digestive factors in Nile tilapia (Oreochromis niloticus) larvae. Aquaculture, 735545.

Fabrikov, D., Sánchez-Muros, M. J., Barroso, F. G., Tomás-Almenar, C., Melenchón, F., Hidalgo, M. C., ... & Montes-Lopez, J. (2020). Comparative study of growth performance and amino acid catabolism in Oncorhynchus mykiss, Tinca tinca and Sparus aurata and the catabolic changes in response to insect meal inclusion in the diet. Aquaculture, 529, 735731.

Food, F. A. O. (2018). Agriculture Organization of the United Nations. 2016. The State of

World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

FAUCONNEAU, B. (1985). Protein synthesis and protein deposition in fish. Nutrition and feeding in fish, 17-45.

FERNANDES, A.F.A., ALVARENGA, É. R., OLIVEIRA, D.A.A., ALEIXO, C.G., PRADO, S.A., LUZ, R.K., TURRA, E.M., 2013. Production of oocytes of Nile tilapia *(Oreochromis niloticus*) for in vitro fertilization via hormonal treatments. Reproduction in Domestic Animal, 48(6), 1049-1055.

Furné, M., Hidalgo, M.C., Lopez, A., Garcia-Gallego, M., MoraleS, A.E., Domezain, A., Domezaine, J., Sanz, A., 2005. Digestive enzyme activities in Adriatic sturgeon Acipenser naccarii and rainbow trout Oncorhynchus mykiss, a comparative study. Aquaculture 250, 391–398.

Guerra-Santos, B., López-Olmeda, J.F., de Mattos, B.O., Baião, A.B., Pereira, D.S.P., Sánchez-Vázquez, F.J., Fortes-Silva, R., 2017. Synchronization to light and mealtime of daily rhythms of locomotor activity, plasma glucose and digestive enzymes in the Nile tilapia (Oreochromis niloticus). Comp. Biochem. Physiol. A 204, 40–47.

JOBLING, M. (1995). Simple indices for the assessment of the influences of social environment on growth performance, exemplified by studies on Arctic charr. Aquaculture International, 3(1), 60-65.

Kazimi, N., & Cahill, G. M. (1999). Development of a circadian melatonin rhythm in embryonic zebrafish. Developmental Brain Research, 117(1), 47-52.

KOUMOUNDOUROS, G., SFAKIANAKIS, D. G., DIVANACH, P., & KENTOURI, M. (2002). Effect of 36emperatura on swimming performance of sea bass juveniles. Journal of Fish Biology, 60(4), 923-932.

KULCZYKOWSKA, E.; POPEK, W.; KAPOOR, B.G. Biological clock in fish. New York: CRC Press, 2010. p.280.

Lahiri, K., Froehlich, N., Heyd, A., Foulkes, N.S., Vallone, D., 2014. Developmental stagespecific regulation of the circadian clock by temperature in zebrafish. Biomed. Res.Int. 2014, 930308.

Luz, R.K., Melillo Filho, R., Santos, A.E.H., Rodrigues, L.A., Takata, R., Alvarenga, É.R.D., Turra, E.M., 2012. Stocking density in the larviculture of Nile tilapia in saline water. R. Bras. Zootec. 41, 2385–2389.

LIVAK, K. J., & SCHMITTGEN, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods, *25*(4), 402-408.

LÓPEZ-OLMEDA, J. F., & SÁNCHEZ-VÁZQUEZ, F. J. (2011). Thermal biology of zebrafish (*Danio rerio*). Journal of Thermal Biology, 36(2), 91-104.

López-Olmeda, J.F., 2017. Nonphotic entrainment in fish. Comp. Biochem. Physiol. A 203, 133–143.

López-Olmeda, J.F., López-García, I., Sánchez-Muros, M.J., Blanco-Vives, B., Aparicio, R., Sánchez-Vázquez, F.J., 2012. Daily rhythms of digestive physiology, metabolism and behaviour in the European eel (Anguilla anguilla). Aquac. Int. 20, 1085–1096.

Ma, H., Wei, P., Li, X., Liu, S., Tian, Y., Zhang, Q., & Liu, Y. (2021). Effects of photoperiod on growth, digestive, metabolic and non-special immunity enzymes of Takifugu rubripes larvae. Aquaculture, 736840.

Martín-Robles, Á.J., Whitmore, D., Pendon, C., Munoz-Cueto, J.A., 2013. Differential effects of transient constant light-dark conditions on daily rhythms of Period and Clock transcripts during Senegalese sole metamorphosis. Chronobiol. Int. 30, 699–710.

Mata-Sotres, J.A., Moyano, F.J., Martínez-Rodríguez, G., Yúfera, M., 2016. Daily rhythms of digestive enzyme activity and gene expression in gilthead seabream (Sparus aurata) during ontogeny. Comp. Biochem. Physiol. A 197, 43–51.

MOURA, G., OLIVEIRA, M. G. A., & LANNA, E. (2012). Desempenho e atividade de lipase em tilápias do Nilo. Archivos de zootecnia, 61(235), 367-374.

Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J., 2010. Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (Sparus aurata). Aquaculture 306, 315–321.

Navarro-Guillén, C., Moyano, F.J., Yúfera, M., 2015. Diel food intake and digestive enzyme production patterns in Solea senegalensis larvae. Aquaculture 435, 33–42.

NASCIMENTO, FLÁVIO LIMA; NAKATANI, KESHYIU. (2006). Relações entre fatores ambientais e a distribuição de ovos e larvas de peixes na sub-bacia do rio Ivinhema, Estado de Mato Grosso do Sul, Brasil. Acta Scientiarum. Biological Sciences, 28, 2, 117-122.

Ndiwa, T. C., Nyingi, D. W., Claude, J., & Agnèse, J. F. (2016). Morphological variations of wild populations of Nile tilapia (Oreochromis niloticus) living in extreme environmental conditions in the Kenyan Rift-Valley. Environmental Biology of Fishes, 99(5), 473-485.

Omondi, R., Kembenya, E., Nyamweya, C., Ouma, H., Machua, SK, & Ogari, Z. (2014). Mudanças limnológicas recentes e suas implicações na pesca no Lago Baringo, Quênia.

OKAMOTO, MARCELO HIDEO; DE SAMPAIO, LUÍS ANDRÉ NASSR; DE PINHO MAÇADA, ARMINDO. (2006) Efeito da temperatura sobre o crescimento e a sobrevivência de juvenis da tainha *Mugil platanus* Günther, 1880. Atlântica (Rio Grande), 28 (1) 61-66, 2006.

OSPINA-ALVAREZ, NATALIA; PIFERRER, FRANCESC. (2008). Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS one, 3, 2837.

PANKHURST, N. W., & MUNDAY, P. L. (2011). Effects of climate change on fish reproduction and early life history stages. Marine and Freshwater Research, 62(9), 1015-1026.

PANKHURST, N. W; KING, H. R. (2010). Temperatura e reprodução de salmonídeos: implicações para a aquicultura. Journal of Fish Biology,76(1), 69-85.

PEREZ-CASANOVA, J. C., MURRAY, H. M., GALLANT, J. W., ROSS, N. W., DOUGLAS, S. E., & JOHNSON, S. C. (2006). Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture, 251(2-4), 377-401.

Patterson, G., Wilson, K.K., 1995. The influence of the diel climatic cycle on the depthtime distribution of phytoplankton and photosynthesis in a shallow equatorial lake (Lake Baringo, Kenya). Hydrobiologia 304, 1–8.

PRIETO-GUEVARA, M.J.; SILVA, R.F.; COSTA, L.S. (2015). Effect of fixed feeding time on growth, body composition, and hepatic histology of hybrid catfish (*Pseudoplatystoma reticulatum x Leiarius marmoratus)* fed with carbohydrates and lipids ratios. Revista Colombiana de Ciências da Pesca,28, 83-95.

Qiang, J., He, J., Yang, H., Sun, Y.L., Tao, Y.F., Xu, P., Zhu, Z.X., 2017. Dietary lipid requirements of larval genetically improved farmed tilapia, Oreochromis niloticus (L.), and effects on growth performance, expression of digestive enzyme genes, and immune response. Aquac. Res. 48, 2827–2840.

ROMBOUGH, P. J. (1997, January). The effects of temperature on embryonic and larval development. In Seminar Series-Society For Experimental Biology (Vol. 61, pp. 177-224).Cambridge University Press.

SÁNCHEZ-VÁZQUEZ, F. J., & LÓPEZ-OLMEDA, J. F. (2018). Environmental cycles and biological rhythms during early development. In Emerging Issues in Fish Larvae Research (pp. 37-50). Springer, Cham.

SÁNCHEZ-VÁZQUEZ, F.J.; ARANDA, A.; MADRID, J.A. (2001). Differential effects of meal size and food energy density on feeding entrainment in goldfish. Journal of Biological Rhythms, 16, 58-65.

SCHAEFER, J., & RYAN, A. (2006). Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. Journal of fish biology, 69(3), 722-734.

SILVA, W. S., COSTA, L. S., LÓPEZ-OLMEDA, J. F., COSTA, N. C. S., SANTOS, W. M., RIBEIRO, P. A. P., & LUZ, R. K. (2019). Gene expression, enzyme activity and performance of Nile tilapia larvae fed with diets of different CP levels. *animal*, *13*(7), 1376-1384.

TENGJAROENKUL, B., SMITH, B. J., SMITH, S. A., & CHATREEWONGSIN, U. (2002). Ontogenic development of the intestinal enzymes of cultured Nile tilapia, *Oreochromis niloticus L.* Aquaculture, 211(1-4), 241-251.

UNTERGASSER, A., CUTCUTACHE, I., KORESSAAR, T., YE, J., FAIRCLOTH, B. C., REMM, M., & ROZEN, S. G. (2012). Primer3—new capabilities and interfaces. Nucleic acids research, 40(15), e115-e115.

VILLAMIZAR, N., BLANCO-VIVES, B., MIGAUD, H., DAVIE, A., CARBONI, S., & SANCHEZ-VAZQUEZ, F. J. (2011). Effects of light during early larval development of some aquacultured teleosts: a review. Aquaculture, 315(1-2), 86-94.

VILLAMIZAR, N., RIBAS, L., PIFERRER, F., VERA, L.M., SÁNCHEZ-VÁZQUEZ, F.J., (2012). Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. PLoS ONE 7(12), e52153.

VOLPATO, G.L. & TRAJANO, E. Biological Rhythms- Fish physiology. San Diego: Elsevier, (2006). p.101-153. Vera, L.M., De Pedro, N., Gomez-Milan, E., Delgado, M.J., Sanchez-Muros, M.J., Madrid, J.A., Sanchez-Vazquez, F.J., 2007. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiol. Behav. 90, 518–524.

Zeytin, S., Schulz, C., Ueberschär, B., 2016. Diurnal patterns of tryptic enzyme activity under different feeding regimes in gilthead sea bream (Sparus aurata) larvae. Aquaculture 457, 85–90.

Ziv, L., Gothilf, Y., 2006. Circadian time-keeping during early stages of development.Proc. Natl. Acad. Sci. 103, 4146–4151.

8. Considerações finais

Animais mantidos em uma condição cíclica de temperatura maturam e sincronizam precocemente suas enzimas digestivas e metabólicas, além de maturar o sistema endócrino, envolvido diretamente no crescimento animal. Essa maturação precoce de enzimas e hormônios leva a um aumento do crescimento, como foi demonstrado no presente estudo.

O melhor aproveitamento da dieta gera, consequentemente, melhorias no desempenho, proporcionando redução nos custos com alimentação, bem como redução no período de criação dos animais.

A maior compreensão da dinâmica fisiológica em larvas de tilápia melhora os índices produtivos, assim como proporciona ajustes nos protocolos de manejo nas primeiras fases de desenvolvimento dos animais.

Estudos futuros sobre a influência de termociclos no desenvolvimento de tilápias devem ser realizados para as demais fases de cultivo.

ANEXOS

Anexo 1- Artigo publicado

Aquaculture 528 (2020) 735545

Contents lists available at ScienceDirect

Aquaculture

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

Effects of temperature regime on growth and daily rhythms of digestive factors in Nile tilapia (*Oreochromis niloticus*) larvae

Amanda Hastenreiter Espirito Santo[a], Gonzalo de Alba[b], Yane da Silva Reis[c], Leandro Santos Costa[d], Francisco Javier Sánchez-Vázquez[b], Ronald Kennedy Luz[a], Paula Adriane Perez Ribeiro[a], Jose Fernando López-Olmeda[b,*]

[a] *Departamento de Zootecnia, Universidade Federal de Minas Gerais, Escola de Veterinária, Laboratório de Aquacultura, CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[b] *Department of Physiology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain*
[c] *Centro de Ciências Agrícolas, Ambientais e Biológicas (CCAAB), Campus Cruz das Almas, Universidade Federal da Bahia (UFRB), 44380-000 Bahia, Brazil*
[d] *Departamento de Zootecnia, Universidade Federal Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil*

ARTICLE INFO

Keywords:
Biological clock
Larviculture
Fish development
Digestive function
Endocrine system

ABSTRACT

Environmental factors such as light and temperature present cyclic variations during the day. High temperatures occur during the light phase, whereas low temperatures take place at night (*i.e.* a daily thermocycle). Although applying thermocycles to fish larvae improves growth in some species, nothing is known about the impact during the early development of the digestive system. The aim of the present research was to investigate the effect of different temperature regimes, cycling *versus* constant, on the daily rhythms of digestive factors and growth of Nile tilapia (*Oreochromis niloticus*) larvae. For this purpose, fertilized eggs (stage 1) were divided into two groups: one under a thermocycle (TC) of 31 °C:25 °C day:night and another group at constant temperature of 28 °C (CTE). Photoperiod was set at 12:12 h light/dark cycle. Larvae length was measured on 4, 8 and 13 days post-fertilization (dpf) and samples were collected every 4 h during a 24-h cycle on these days. The expression levels of *pepsinogen*, *chymotrypsinogen*, *trypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck* were analyzed by qPCR. The results showed that growth was greater when larvae were reared at TC than at CTE. Moreover, on 13 dpf, most analyzed genes (*chymotrypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck*) displayed daily rhythms in the TC group but not in CTE, with most acrophases located around mealtime. These rhythms might explain the higher growth rate observed in the TC larvae due to improved synchronization of feed digestion and utilization. These findings could be useful for improving rearing protocols used in tilapia larviculture and enhancing production performance.

1. Introduction

The natural environment presents cyclic and predictable variations in geophysical variables, such as light and temperature. These cyclic oscillations have influenced evolution and selected the occurrence of biological clocks in most living organisms. Biological rhythms confer adaptive advantages as organisms can time their physiological processes to occur at specific moments when effectiveness is the greatest (DeCoursey, 2004). Among the environmental variables that synchronize biological rhythms, light has been commonly regarded as the most important but other factors, such as temperature and feeding, play an important role, especially in ectothermic animals like fish (López-Olmeda, 2017).

Temperature has a strong influence on fish behavior and physiology (Brett, 1971) as it affects processes like development, locomotion, sex differentiation, reproduction and survival (Bennett and Beitinger, 1997; Ospina-Álvarez and Piferrer, 2008; Pankhurst and King, 2010; López-Olmeda and Sánchez-Vázquez, 2011). In the natural environment, daily temperature oscillations (thermocycles) are generated in the water due to the presence or absence of solar infrared radiation (Villamizar et al., 2011). Hence temperature displays cycles alongside the light/dark cycle, with the higher temperature phase (thermophase) coinciding with day and the lower temperature phase (cryophase) coinciding with night (López-Olmeda and Sánchez-Vázquez, 2011). However, while most fish undergo thermocycles in the wild, their effects on fish biology, especially during early development, have received little

* Corresponding author.
E-mail address: jflopez@um.es (J.F. López-Olmeda).

https://doi.org/10.1016/j.aquaculture.2020.735545
Received 17 February 2020; Received in revised form 25 May 2020; Accepted 25 May 2020
Available online 27 May 2020
0044-8486/ © 2020 Elsevier B.V. All rights reserved.

attention to date (Schaefer and Ryan, 2006; Villamizar et al., 2011). Thermocycles during larval development have been reported to increase survival and growth, lower the incidence of malformations and modify the sex ratio (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018).

Nile tilapia (*Oreochromis niloticus*) is a species that tolerates a relatively broad range of temperatures and is subjected to wide temperature variations in the wild, from temperate to warm waters (Omondi et al., 2014; Ndiwa et al., 2016). For instance, in one of its natural environments, the lakes of the Great Rift Valley in Africa, air temperature shows wide daily fluctuations (Crul, 1995), which generate daily variations in water temperature of several degrees of amplitude, *i.e.* 24–30 °C (min–max) (Patterson and Wilson, 1995). Moreover, although temperature is controlled in many culture systems used for tilapia (Boyd, 2004), similar variations may occur in extensive open systems such as ponds or net cages located in rivers, where water temperature can display daily variations of up to 5–6 °C (Culberson and Piedrahita, 1996; Jomori et al., 2003; Lopes and Henry-Silva, 2014). However, despite the importance of the variations in water temperature in its natural habitat, this topic has received little consideration in previous studies on tilapia development (Azaza et al., 2010)

One of the most important factors to affect the efficiency of food conversion into nutrients is the availability of the digestive enzymes involved in this process (Jobling, 1995; Perez-Casanova et al., 2006). The maximum fish growth rate, especially during early development, strongly depends on digestive capacity (Blier et al., 1997). Thus research on the ontogeny of digestive enzymes in fish can elucidate certain aspects of their nutritional physiology and help to solve nutritional challenges in larviculture (Furné et al., 2005). In fish, studies on the ontogeny of the digestive function at the molecular level are scarce. In addition, studies on the existence of rhythms of digestive factors during development and how the environment modulates these rhythms are lacking (Rønnestad et al., 2013; Yúfera et al., 2018). Studying the existence of daily rhythms in larval physiology is also crucial because most research has usually focused only on one single daily sample, neglecting daily oscillations (Yúfera et al., 2018). To date, the existence and ontogeny of these rhythms in fish larvae have only been reported in two marine species: gilthead sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016).

The larviculture of Nile tilapia offers some advantages, such as using formulated diets from the start of exogenous feeding (Luz et al., 2012). However, for this species, more in-depth knowledge about the digestive physiology in the larval stage is necessary (De Moura Pereira et al., 2019). To date, research into the ontogeny of digestive factors during larval development is scarce, and only a few reports have addressed this topic (Tengjaroenkul et al., 2002; Qiang et al., 2017; De Moura Pereira et al., 2019; Silva et al., 2019). Daily rhythms in clock gene expression and factors from the endocrine axis have been reported in adult Nile tilapia (Costa et al., 2016a, 2016b; de Alba et al., 2019), but still nothing is known about the existence of the daily rhythms in digestive factors for tilapia larvae.

Therefore, the aim of the present study was to evaluate the effects of two different temperature regimes, a daily thermocycle *versus* constant temperature, applied on the first days of Nile tilapia development, on the growth and daily rhythms of different digestive physiology parameters. These parameters involved the expression of genes that encode enzymes for protein (*pepsinogen*, *chymotrypsinogen* and *trypsinogen*), lipid (*lipase*) and carbohydrate digestion (*maltase* and *isomaltase*), and the endocrine control of food intake and digestion (*neuropeptide Y*, *npy*, and *cholecystokinin*, *cck*).

2. Materials and methods

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia (Spain). Fish were reared following the Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals. In addition, the protocols were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

2.1. Animals and experimental design

Male and female Nile tilapia (*Oreochromis niloticus*) adults were obtained from a local fish farm (Tilamur S.A., Murcia, Spain). Animals were kept in 300-l tanks connected to a recirculation system, equipped with biological and mechanical filters. The photoperiod was set at 12:12 h light/dark (LD) cycle with lights on at 09:00 h. The water temperature was maintained controlled at 28 ± 0.5 °C. Adult tilapias were fed a commercial diet (D-4 AlternaBasic 2P, Skretting, Spain) with 36% crude protein (CP) 3 times a day until apparent satiety.

After acclimation, tilapia breeders were induced with human Chorionic Gonadotropin hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013). Briefly, females were intraperitoneally (i.p.) injected with 1500 IU/kg of body weight and males with 500 IU/kg. The administration for females was divided into two i.p. injections: the first consisted of 500 IU/kg injected at the end of the light phase and the second one (1000 IU/kg) was applied 12 h after the first dose (at the beginning of the light phase on the next day). Males received a single i.p. injection at the same time as the second dose for females. After hCG administration, animals were placed together. After 24 h, sperm and eggs were collected by stripping and *in vitro* fertilization was performed (Fernandes et al., 2013). This procedure allowed us to immediately obtain fertilized eggs, which were used in the experiments.

Fertilized eggs were obtained from five different groups of tilapia breeders (3 females and 5–6 males per group). Then eggs at stage 1 (Fujimura and Okada, 2007) were pooled together and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (150–200 eggs per incubator, 6 incubators per group were used) in two distinct systems with different temperature regimes: one system with a daily thermocycle (TC) of 31 °C:25 °C, and another with a constant temperature (CTE) of 28 °C. Both systems were recirculating water systems that were connected to mechanical and biological filters. The photoperiod was set at 12:12 LD, with lights on at 09:00 h. By convention, in chronobiological studies, the time of light onset is set as *Zeitgeber* (synchronizer) time 0 h (ZT0 h), in order to standardize time points regardless local time, facilitating reproducibility (Guerra-Santos et al., 2017). Hence, light onset at 9:00 h corresponds to ZT 0 h and light offset at 21:00 h corresponds to ZT 12 h. In the TC system, the thermophase (high temperature phase) of 31 °C coincided with the light phase, whereas the cryophase (low temperature) of 25 °C coincided with the dark phase (Suppl. Fig. 1A). Temperature difference between the thermo- and the cryophase was selected following previous literature and the normal daily ranges experienced by tilapia in the wild (Patterson and Wilson, 1995; Schaefer and Ryan, 2006; Villamizar et al., 2012; Ndiwa et al., 2016). The average water temperature in the TC system throughout the day was 28.0 °C. Thus the larvae reared in this system were subjected to the same degree days as the animals reared in the CTE group, at an average water temperature of 28.0 ± 0.3 °C (mean ± SD). In the TC system, water temperature was modified by water heaters (Askoll, Povolaro, Italy) and coolers (Aqua-Medic 1500, Titan GmbH, Bissendorf, Germany), controlled by electronic timers (Bachmann GmbH & Co, Stuttgart, Germany). Water temperature was recorded continuously throughout the experiment by an underwater data recorder (HOBO pendant, Onset Computer Corporation, Bourne, MA, USA). Hatching time (day) and rate were recorded for posterior analysis on the influence of the thermal regime on these parameters. Larvae were reared in incubators until 7 days post

fertilization (dpf), when they were transferred to 9-l tanks connected to the same temperature system. Density in the tanks was of approximately 10 larvae/l, and a total of ten 9-l tanks were used in the experiments (5 tanks per temperature treatment). Exogenous feeding began on 7 dpf. Larvae were fed a semi-purified diet containing 42% CP and 4100 Kcal/kg, formulated as described elsewhere (Silva et al., 2019). Larvae were fed in excess 4 times a day in the first half of the light phase (ZT 1, ZT 3, ZT 5 and ZT 7 h) (Suppl. Fig. 1B). Tilapia embryo/larvae were maintained under the experimental conditions from 0 to 13 dpf.

A total of 1014 tilapia larvae were used in the experiments: 864 larvae for qPCR analyses and 150 larvae for total length measures. On 4, 8 and 13 dpf, whole larvae samples were collected every 4 h during a 24-h cycle at the following time points: ZT 2, ZT 6, ZT 10, ZT 14, ZT 18 and ZT 22 h (Suppl. Fig. 1B). Larvae were pooled for each replicate and six replicates ($n = 6$) were collected for each group, time point and day. Larvae were stored in 1.5 ml tubes and immediately frozen and stored at −80 °C until analyzed. The number of larvae used in the pool differed depending on the sampling day: 5 larvae/pool on 4 dpf; 4 larvae/pool on 8 dpf; 3 larvae/pool on 13 dpf. The larvae collected on 8 and 13 dpf were fed on the sampling day.

2.2. Larval growth

The growth of the tilapia larvae in both temperature groups (TC and CTE) was evaluated on 4, 8 and 13 dpf. For this purpose, 75 larvae were used for each treatment, and 25 larvae were used for each measurement day. Larvae were removed from the aquarium and transferred to a Petri dish. Then each larvae was photographed using a binocular (Leica EZ4 HD, Leica Microsystems GmbH, Wetzlar, Germany) with an incorporated digital camera. Photos were stored in a computer and analyzed later with the ImageJ image processing software (Abramoff et al., 2004). Larvae were measured longitudinally by considering standard length (Suppl. Fig. 2).

2.3. Real-time RT-PCR analysis

Larvae pools were transferred to sterile tubes and homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions, using a tissue homogenizer for mechanical homogenization (TissueLyser LT, Qiagen, Hilden, Germany). RNA was dissolved in DEPC water (Invitrogen, Carlsbad, USA) and RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Total RNA (1 μg) was first treated with 1 U of DNase (Dnase I, Thermo Fisher), followed by retrotranscription using a commercial kit (qScript cDNA Synthesis Kit, Quantabio, Beverly, USA). The quantitative PCR (qPCR) reactions were performed by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). All the samples were run in duplicate and the qPCR reactions were performed in a final volume of 20 μl. The quantitative PCR analyses were performed in a light thermocycler (7500 RT-PCR system, Applied Biosystems) following this protocol: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were run after amplification to ensure that only one DNA species was amplified. All the primer sequences (Table 1) were designed with the Primer 3 plus software (Untergasser et al., 2012). The relative amplification efficiencies of all the genes were analyzed by cDNA dilution curves and were found to be similar for all the genes. Primer concentrations were determined by means of a primer dilution curve. The primers for *chymotrypsinogen*, *pepsinogen*, *lipase*, *npy*, *maltase* and *βactin* were added at a reaction concentration of 200 nM. The primers for *trypsinogen*, *isomaltase* and *cck* were added at a final concentration of 400 nM. The relative expression of all the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The reference gene, *βactin*, was selected after verifying that its coefficient of variation (CV) was lower than 5% and was used in the first normalization. The sample with the lowest expression value within each gene, tissue and group was used as the reference for the second normalization.

2.4. Data analysis

All the results are expressed as mean ± SEM. The significance threshold was set at $\alpha = 0.05$ for all the tests. The SPSS software (v. 19.0, IBM, Armonk, USA) was used to detect any statistically significant differences between groups and time points. Normality of the data was previously assessed by the Kolmogorov-Smirnov test and homogeneity of variance was also verified using Levene's test. Data were subjected to a two-way ANOVA, followed by Duncan's *post hoc* test, to check for statistically significant differences in gene expressions between groups (TC *versus* CTE) and sampling points (ZTs), and differences in length between groups and days post fertilization (4, 8 and 13 dpf). Data of hatching rate and hatching day were subjected to a Student's *t*-test and a Mann-Whitney *U* test, respectively.

The existence of significant rhythmicity was tested for all the genes by the Cosinor analysis, performed with the "EL TEMPS" software (v.1.179, Prof. Díez-Noguera, University of Barcelona, Spain). The Cosinor analysis is based on the least squares approach of time series data with a cosine function of a known period of type $Y = \text{Mesor} + \text{Amplitude} * \cos(2\pi(\text{t-Acrophase})/\text{Period})$. The Cosinor analysis also provides the statistical significance of the rhythm by an F-test of the variance, accounted for by the waveform *versus* a straight line of zero-amplitude (null hypothesis).

3. Results

3.1. Hatching timing and rate

The eggs presented a similar hatching rate in the two temperature rearing conditions (*t*-test, $p = .652$). Eggs at TC had a hatching rate of 49.6 ± 13.3% (mean ± SEM) whereas eggs at CTE presented a rate of 50.5 ± 11.6%. Regarding hatching time, all eggs hatched between 2 and 4 dpf, with most hatching events occurring at days 2 and 3. Eggs at TC hatched mainly at 2 dpf (2.3 ± 0.3 dpf, mean ± SEM) whereas eggs at CTE hatched mainly at 3 dpf (3.3 ± 0.3 dpf), but these differences were not statistically significant (Mann-Whitney U test, $p = .114$).

3.2. Larval growth

The length was significantly influenced by the developmental stage (dpf) (two-way ANOVA, $p < .001$), the temperature regime in which tilapia embryo and larvae were raised ($p = .001$) and the interaction between this two factors ($p < .001$) (Fig. 1). In both temperature treatments, differences were observed between 4, 8 and 13 dpf, with larval length significantly increasing from one developmental stage to the following one (Fig. 1). In addition, significant differences were observed between groups at 13 dpf. By this day, the TC treatment larvae presented significantly higher length values (8.09 ± 0.07 mm, mean ± SEM) compared to those in the CTE treatment (7.77 ± 0.09 mm) (Fig. 1).

3.3. Pepsinogen expression

No daily rhythms (Cosinor, $p = .068$–0.345) were found for *pepsinogen* relative expression in the 4, 8 or 13 dpf tilapia larvae in either of the tested temperature regimes (Fig. 2). Statistically significant effects were observed for time at 4 dpf and group at 13 dpf (two-way ANOVA, $p = .001$ and 0.026, respectively) (Suppl Table 1). In addition, a significant effect in the interaction of both factors (time and group) was detected at 4 and 13 dpf ($p = .002$ and 0.041, respectively) (Fig. 2A and C). At 4 dpf, *pepsinogen* expression in TC at ZT 2 h was significantly higher than CTE at the same time and other time points in the TC group

Table 1
Primer sequences used for the quantitative analysis by qPCR.

Gene	F/R	Sequence (5′-3′)	Amplification efficiency (%)	Ensembl/GenBank accession number
pepsinogen	F	TGACCAATGACGCTGACTTG	96.49	JQ043215.1
	R	GGAGGAACCGGTGTCAAAAATG		
chymotrypsinogen	F	TTCTGCCTTCGCTTCTCATC	101.95	ENSONIG00000003237
	R	TTCAACGCCATCTGCTACTG		
trypsinogen	F	AGTGCGCAAAGAACTCTGTG	101.65	AY510093.1
	R	AATGTTGTGCTCACCAAGGC		
lipase	F	CTACAACTGCTCCACCAGCA	95.17	NM_001279753.1
	R	GGTGTAGTCGGTGAGCCATT		
maltase	F	ACGGTGGAATCACAGGACTC	97.71	XM_005459498.4
	R	GAAGGCTGCTGATGTGTTCA		
isomaltase	F	GGATCATTCTTCTGGGACGA	103.98	XM_003441717.4
	R	AGGTTGTGCTGTGGGGTTAG		
cck	F	AGAAACTCCACGGCAAACAG	96.30	ENSONIG00000019439.1
	R	ACTCATACTCCTCTGCACTGC		
npy	F	ACACCCAACACTGCTTGAAG	102.21	ENSONIG00000004499
	R	TGTTGCACAGATGACGACTC		
βactin	F	TGGTGGGTATGGGTCAGAAAG	98	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA		

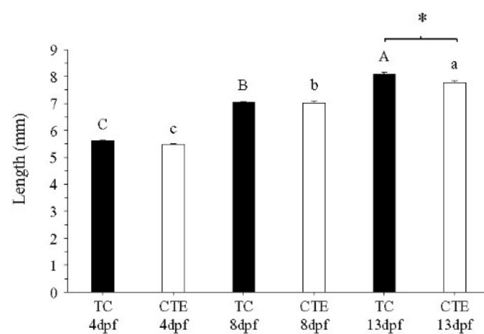


Fig. 1. Standard length (mm) of tilapia larvae on 4, 8 and 13 days post fertilization (dpf) at a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (black bars) or constant temperature (CTE) of 28 °C (white bars). Different upper case and lower case letters indicate statistically significant differences between developmental stages (dpf) within the TC or CTE groups, respectively (two-way ANOVA). The asterisk indicates significant differences between groups in the same dpf (two-way ANOVA). Data are represented as mean ± SEM; $n = 25$ replicates (larva) per point.

(Fig. 2A). In addition, on 13 dpf, *pepsinogen* levels in CTE at ZT 2 h were higher than TC at the same time and other time points in the CTE group (Fig. 2C).

3.4. Chymotrypsinogen expression

The relative expression of *chymotrypsinogen* displayed significant daily rhythms for the 4 dpf larvae maintained in the CTE group (Cosinor, $p = .018$), on 8dpf in both treatments ($p = .024$ and $p = .007$ for TC and CTE, respectively) and for the 13 dpf larvae maintained in TC ($p = .044$) (Fig. 3). In addition, the acrophases varied between the significant detected rhythms (Table 2). In the CTE group, acrophases were located in the dark phase at ZT 18:06 h and ZT 21:35 h for 4 and 8 dpf, respectively. In the TC group, the acrophase was located at the beginning of the dark phase on 8 dpf (ZT 14:02 h) and shifted to the beginning of the light phase on 13 dpf (ZT 02:36 h). Moreover, *chymotrypsinogen* expression in the 8 dpf larvae showed significant differences depending on the groups (two-way ANOVA, $p = .002$) and in the interaction between time and group factors ($p = .007$) (Fig. 3B and Suppl Table 1). At 8 dpf, *chymotrypsinogen* in the TC group showed significant variations between time points, with higher expression at ZT 14 h than ZT 2, 6 and 22 h (Fig. 3B). The expression was also higher in TC at ZT 10 and 14 h compared with the CTE group (Fig. 3B).

3.5. Trypsinogen expression

The relative expression of *trypsinogen* in tilapia larvae showed significant daily rhythms in the larvae raised in TC on both 4 and 8 dpf (Cosinor, $p = .029$ and $p = .025$, respectively) (Fig. 4). The acrophase of *trypsinogen* for the 4 dpf TC was located at the beginning of the light phase (ZT 03:10 h) and shifted to the end of the light phase in the 8 dpf larvae (ZT 10:11 h) (Table 2). In addition, *trypsinogen* in the 4 dpf larvae displayed significant differences depending on the group (two-way ANOVA, $p = .002$), the time of the day ($p < .001$) and the interaction between these two factors ($p < .001$) (Fig. 4A and Suppl Table 1). At this stage, *pepsinogen* expression in TC at ZT 2 h was significantly higher than CTE at the same time and other time points in the TC group (Fig. 4A).

3.6. Lipase expression

Lipase expression only displayed significant daily rhythms in the tilapia maintained under the TC conditions on both 4 and 13 dpf (Cosinor, $p = .044$ and $p = .009$, respectively) (Fig. 5). The acrophases in these two stages occurred at similar times, in the first half of the light phase (ZT 03:54 h and 02:47 h on 4 and 13 dpf, respectively) (Table 2). Furthermore, lipase expression showed significant differences depending on the group at 8 dpf (two-way ANOVA, $p < .001$), depending on the time of the day at 4 and 8 dpf ($p = .010$ and 0.019, respectively), and in the interaction of both factors at 4 and 8 dpf ($p = .022$ and $p < .001$, respectively) (Fig. 5 and Suppl Table 1). On 4 dpf, *lipase* levels in the TC group peaked at ZT 2 h and were significantly higher than levels at ZT 10 and 18 h in the same group (Fig. 5A). At this stage, significant differences in lipase between TC and CTE were found at ZT 18 h (Fig. 5A). On 8 dpf, *lipase* expression was higher in the CTE group than the TC group at all time points except ZT 22 h (Fig. 5B). In addition, *lipase* expression showed differences between time points within the CTE group at 8 dpf, with higher levels at ZT 2 h (Fig. 5B).

3.7. Maltase expression

The only significant daily rhythm in *maltase* expression was detected in the 13 dpf larvae from the TC group (Cosinor, $p = .021$) (Fig. 6). In these animals, *maltase* expression peaked around the middle of the dark

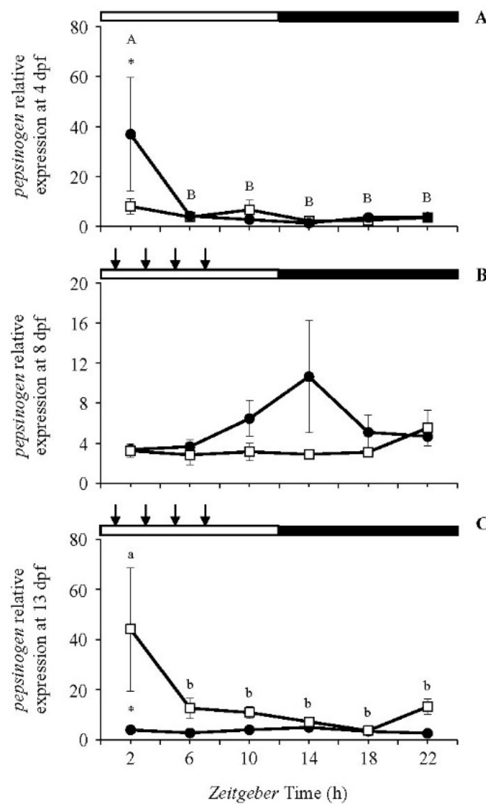


Fig. 2. Daily variations in the relative mRNA expression (fold change) of *pepsinogen* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT0 h corresponds to light onset. Data are represented as mean ± SEM; $n = 6$ replicates (larval pools) per point.

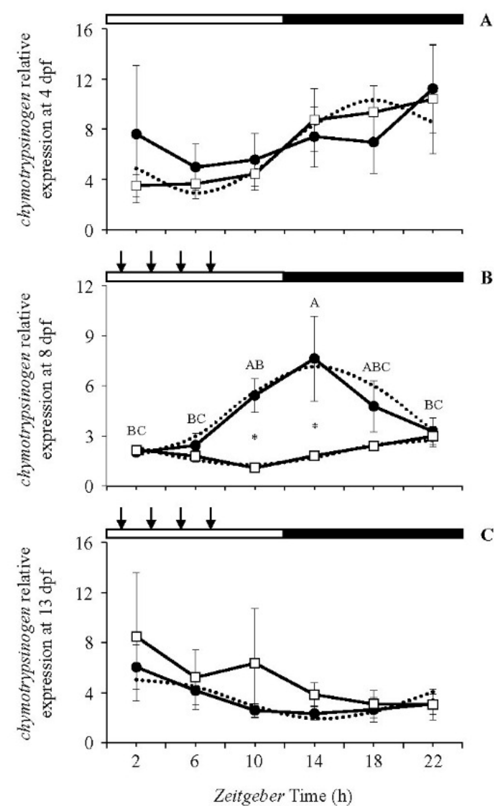


Fig. 3. Daily variations in the relative mRNA expression (fold change) of *chymotrypsinogen* analyzed in tilapia larvae on 4 (A) and 13 (B) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; $n = 6$ replicates (larval pools) per point.

phase, at ZT 17:27 h (Table 2). Moreover, statistically significant differences were found in *maltase* expression depending on the group factor at 8 dpf (two-way ANOVA, $p = .008$). At this stage, *maltase* expression was higher in the CTE than in the TC group (Fig. 6B and Suppl Table 1).

3.8. Isomaltase expression

Significant daily rhythms of *isomaltase* were found at 13 dpf in both groups (Cosinor, $p = .002$ and $p = .042$ for TC and CTE, respectively) (Fig. 7). These rhythms presented opposite acrophases, with *isomaltase* expression peaking around the middle of the dark phase in the TC group (ZT 19:33 h) and about the middle of the light phase in the CTE group (ZT 05:28 h) (Table 2). Significant differences in *isomaltase* expression were observed depending on the group at 8 and 13 dpf (two-way ANOVA, $p = .012$ and 0.006, respectively), and in the interaction between group and time factors ($p = .001$) (Fig. 7 and Suppl Table 1). The overall daily expression of *isomaltase* was higher in CTE than TC at 8 dpf. In addition, on 13 dpf, the TC group showed a higher *isomaltase* expression than CTE at ZT 18 and 22 h (Fig. 7C). The expression of *isomaltase* also showed differences between time points within the TC group at 13 dpf, with higher levels at ZT 18 and 22 h compared with the rest of sampling times (Fig. 7C).

Table 2
Acrophase and statistical significance values of the genes subjected to Cosinor analysis.

Genes	dpf	Acrophase (ZT h)	
		TC	CTE
pepsinogen	4	–	–
	8	–	–
	13	–	–
chymotrypsinogen	4	–	18:06 ± 3:49*
	8	14:02 ± 3:56*	21:35 ± 3:11*
	13	2:36 ± 5:10*	–
trypsinogen	4	3:10 ± 4:52*	–
	8	10:11 ± 4:12*	–
	13	–	–
lipase	4	3:54 ± 5:05*	–
	8	–	–
	13	2:47 ± 3:16**	–
maltase	4	–	–
	8	–	–
	13	17:27 ± 4:03*	–
isomaltase	4	–	–
	8	–	–
	13	19:33 ± 2:08**	5:28 ± 4:09**
npy	4	–	18:38 ± 4:38*
	8	–	–
	13	3:00 ± 3:09**	–
cck	4	–	–
	8	–	–
	13	4:19 ± 3:57*	–

Data are expressed as value ± fiducial limits (set at 95%). The acrophase is indicated in *Zeigeber* Time (ZT) hours. Cosinor *p < .05 **p < .01. Acrophase is not indicated for non-significant genes (p > .05).

3.9. Npy expression

Significant daily rhythms were found for *npy* expression in 4 dpf larvae maintained in CTE (Cosinor, p = .035) and the 13 dpf larvae from the TC group (p = .007) (Fig. 8). Acrophases were located in the middle of the dark phase (ZT 18:38 h) in the 4 dpf CTE larvae and in the first half of the light phase (ZT 03:00 h) in the 13 dpf TC larvae (Fig. 8A and C) (Table 2). The 13 dpf larvae presented significant differences in *npy* expression depending on the group (two-way ANOVA, p < .001), and in the interaction between group and time factors (p < .001) (Fig. 8C and Suppl Table 1). The CTE group showed a higher *npy* expression than TC at ZT 2, 6 and 22 h (Fig. 8C). The expression of *npy* also showed differences between time points within the CTE group at 13 dpf, with higher levels at ZT 22 h compared with ZT 10, 14 and 18 h (Fig. 8C).

3.10. Cck expression

The only significant daily rhythm in *cck* expression was detected in the 13 dpf larvae from the TC group (Cosinor, p = .021) (Fig. 9). The acrophase of this rhythm was located in the first half of the light phase, close to the middle of the day (ZT 04:19 h) (Fig. 9C and Table 2). Statistically significant differences were found in *cck* expression depending on the group factor at 8 dpf (two-way ANOVA, p = .003) (Suppl Table 1). At this stage, the overall *cck* expression was higher in the TC group compared with CTE (Fig. 9B).

4. Discussion

In the present study, the existence of daily rhythms in the mRNA expression of several genes encoding different digestive enzymes and hormones were analyzed in tilapia larvae, as were the effects of temperature regime on the ontogeny of these rhythms. In general, rearing tilapia larvae under a thermocycle improved the occurrence of daily rhythms in these factors as most displayed significant rhythms on 13 dpf, which was not observed in the larvae maintained at constant temperature (Fig. 10). The acrophases (time of maximum value) of the factors involved in protein and lipids digestion, as well as hormones, were located in the morning, at around mealtime, on 13 dpf in the TC group (Fig. 10). The acrophases of the factors involved in carbohydrate digestion were located at night (Fig. 10). This greater number of significant rhythms occurred together with a higher growth as the TC larvae length was longer than that of the CTE group on 13 dpf.

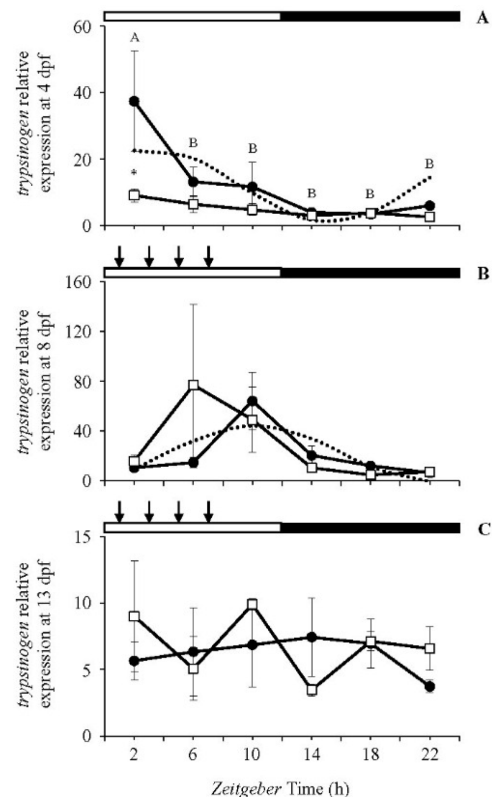


Fig. 4. Daily variations in the relative mRNA expression (fold change) of *trypsinogen* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

When fish develop under cycling conditions, which are similar to the oscillating environmental conditions they experience in nature, they tend to perform better. In a previous study, tilapia juveniles presented

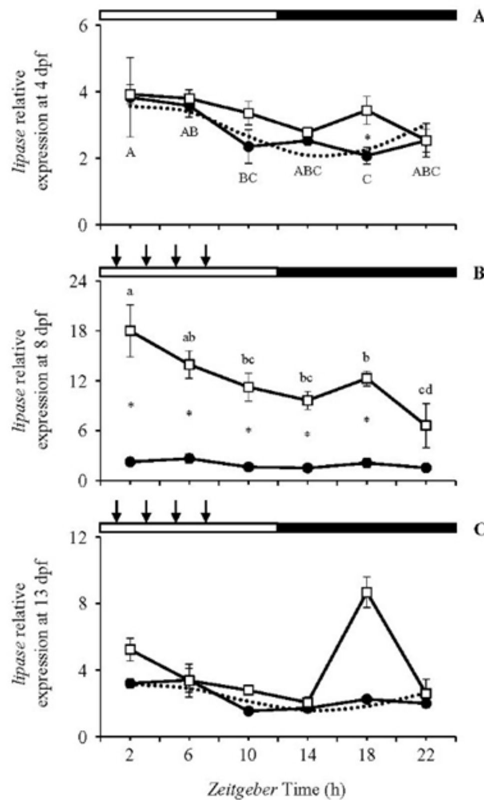


Fig. 5. Daily variations in the relative mRNA expression (fold change) of *lipase* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

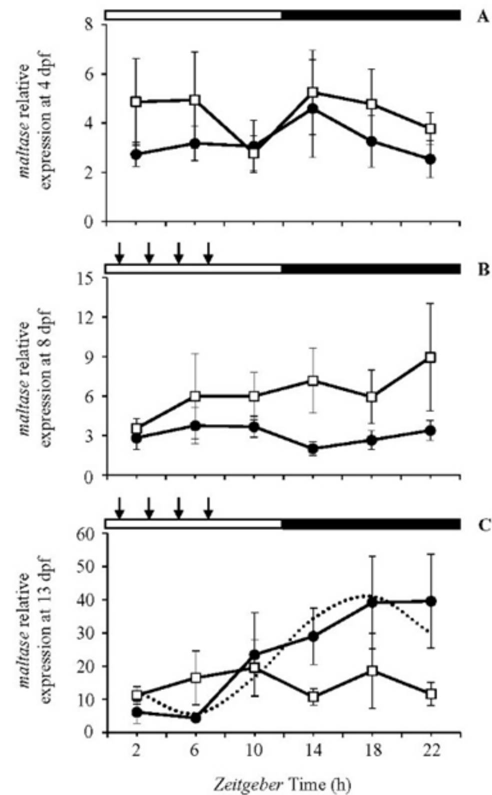


Fig. 6. Daily variations in the relative mRNA expression (fold change) of *maltase* analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

higher growth rates under thermocycles than at constant temperatures, although this effect was lost as fish were becoming bigger and size dispersal was greater (Azaza et al., 2010). In addition, Senegalese sole and zebrafish (*Danio rerio*) present higher growth rates and a lower incidence of malformations when they are kept, during the early development, in thermocycles compared to constant temperatures (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018). This agrees with the results obtained in the present study in tilapia growth with thermocycles. In those previous studies, however, the only analyzed molecular or physiological markers to explain the effects on development induced by thermocycles were some genes involved in sex differentiation as thermocycles also affect sex ratios (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012). Therefore, in the present study, we focused on the analysis of factors involved in food intake control and digestion to investigate whether differences in growth may be explained by differences in these factors.

Regarding factors involved in food intake control, we analyzed *npy* and *cck* mRNA expression. In one hand, Npy is a peptidic hormone mainly produced in the brain (Volkoff, 2006). This hormone is the most potent orexigenic factor in vertebrates, including fish (Narnaware et al., 2000; Delgado et al., 2017). Besides its main effects as a stimulator of food consumption, Npy is a growth hormone (Gh) regulator (Peng and Peter, 1997). On the other hand, Cck is also a peptidic hormone which is synthesized in the brain and gut (Volkoff et al., 2005). Cck is one of the most important regulators of digestion in fish and is largely

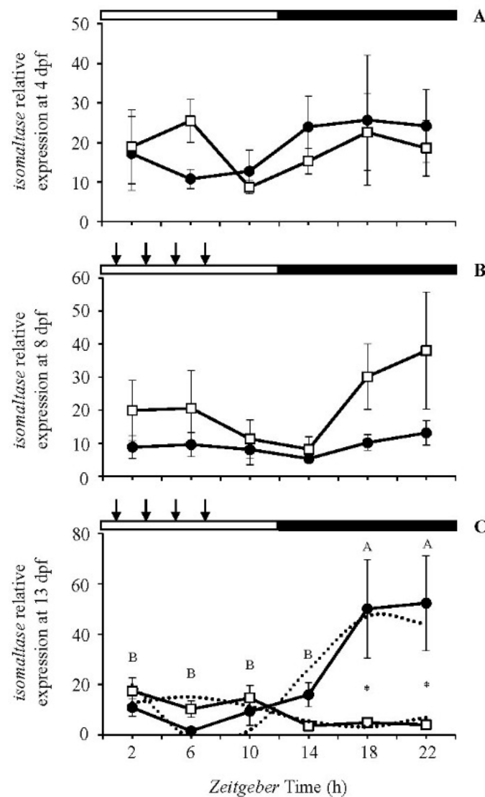


Fig. 7. Daily variations in the relative mRNA expression (fold change) of *isomaltase* analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertilization (dpf) ata 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

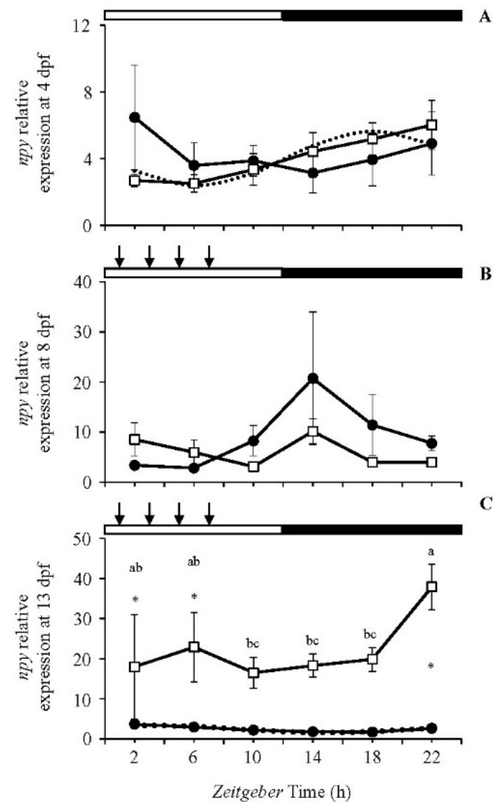


Fig. 8. Daily variations in the relative mRNA expression (fold change) of *neuropeptide Y (npy)* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. Different upper case and lower case letters indicate statistically significant differences between time points within the TC or CTE groups, respectively, on the same graph (two-way ANOVA). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA). The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

responsible for the secretion of pancreatic enzymes (Koven et al., 2002; Volkoff et al., 2005; Zhang et al., 2018). Besides, Cck acts as a satiety (anorexigenic) signal in fish (Volkoff et al., 2005). In the present study, *npy* and *cck* presented rhythms with the highest values at around mealtime in the 13 dpf larvae. These rhythms were observed in larvae reared at TC but not at CTE. Thus one hypothesis that could explain the higher growth in the TC larvae is that the rhythms in these hormones, synchronized with mealtime, might have improved the digestive processes in these larvae compared to the CTE larvae.

Therefore, we analyzed the rhythms of expression of several enzymes involved in digestive processes. The existence of daily rhythms in digestive enzymes in fish was first described in adults (Vera et al., 2007; Montoya et al., 2010; López-Olmeda et al., 2012). In adult tilapia, daily rhythms have been reported in total protease activity, both acid and alkaline (Guerra-Santos et al., 2017). In recent years, the ontogeny of rhythms in digestive factors has been reported in the larvae of two marine species: gilthead sea bream and Senegalese sole (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016; Zeytin et al., 2016). In both species, most of the larval stages analyzed had already started the exogenous feeding and, under these conditions, daily rhythms in digestive enzymes correlated with feeding (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016). This is similar to the result herein found for

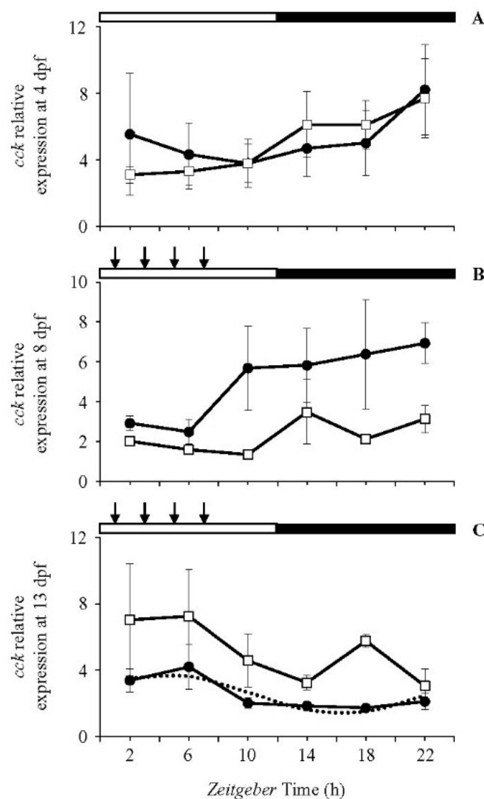


Fig. 9. Daily variations in the relative mRNA expression (fold change) of *cholecystokinin* (*cck*) analyzed in tilapia larvae on4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C (represented by black circles, ●) or constant temperature (CTE) of 28 °C (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant. The white and black bars above each graph represent the light phase and dark phase, respectively. The arrows above the graphs from 8 and 13 dpf indicate feeding times. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset. Data are represented as mean ± SEM; n = 6 replicates (larval pools) per point.

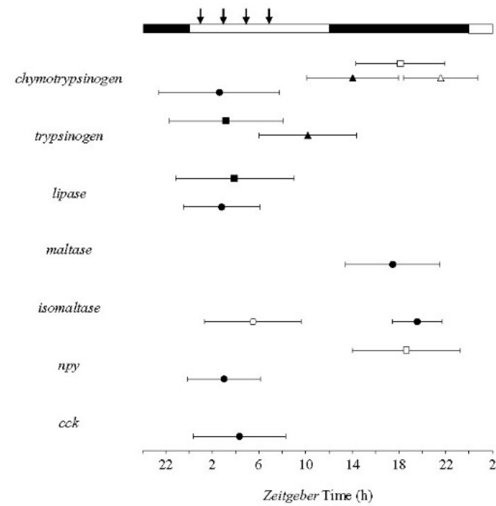


Fig. 10. Map of the acrophases of the digestive factors and hormones analyzed in tilapia larvae from 4, 8 and 13 days post fertilization (dpf) at a 12:12 LD cycle and by two different temperature regimes: a thermocycle (TC) of 31 °C:25 °C or constant temperature (CTE) of 28 °C. The acrophases from the larvae reared in the TC group on 4, 8 and 13 dpf are represented by black squares, triangles and circles, respectively. The acrophases from the larvae reared in the CTE group on 4, 8 and 13 dpf are represented by white squares, triangles and circles, respectively. The acrophase is indicated only for the statistically significant rhythms (Cosinor, $p < .05$). The name of each represented gene is indicated on the left. The white and black bars above the graph represent the light and dark phases, respectively. The arrows above the graph indicate feeding times for 8 and 13 dpf groups. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset.

the TC larvae on 13 dpf, where proteases (*chymotrypsinogen* and *trypsinogen*) and *lipase* presented the highest values around feeding time. The food digestion process depends, among other factors, on the presence and availability of adequate digestive enzymes throughout the gastrointestinal tract (Gisbert et al., 2013). The enzymes observed to display rhythms in tilapia larva are among the most important for protein (trypsin and chymotrypsin) and lipid digestion (lipase acting to release fatty acids and glycerol) (Almeida et al., 2018; Durigon et al., 2019). Taken together, the rhythms in both digestive enzymes and hormones might have increased the efficiency of physiological processes, leading to improved efficiency of food intake, digestion and growth in the TC larvae compared with the CTE group.

Moreover, *maltase* and *isomaltase* presented shifted acrophases and peaked at nighttime. These genes encode for enzymes that act in the final steps of the digestion of dietary carbohydrates (Tengjaroenkul et al., 2002). The peaks of maltase and isomaltase could have occurred later than other enzymes given the process phase in which they are involved. In addition, carbohydrate digestibility is generally poor in fish, although omnivorous fish like tilapia have better digestion rates (Moon, 2001; Kamalam et al., 2017). Thus one possibility could be that carbohydrates remained for longer in the gut and, hence, the expression of these enzymes was delayed. On the other hand, gene expression does not necessarily correlate with the activity of the encoded enzyme (Yúfera et al., 2018), which could be the case for *maltase* and *isomaltase* and would explain why their rhythms are shifted compared to mealtime and to the other analyzed factors. Indeed, a lack of correlation between mRNA expression and the activity of the enzyme encoded should not be discarded for other genes analyzed in the present study. Thus, further research is required concerning enzymatic activity.

Previous studies have highlighted the importance of maintaining embryos/larvae under rearing conditions that are similar to the natural conditions that animals experience in the wild. This mainly involves using environmental cycles instead of constant conditions. For instance, it has been reported that LD cycles are required for correct circadian clock maturation in fish, and constant lighting conditions (either darkness or light) can delay or even suppress the appearance of daily rhythms (Ziv and Gothilf, 2006; Martín-Robles et al., 2013; Cuesta et al., 2014; Di Rosa et al., 2015). The temperature factor has been less studied than light. In zebrafish, thermocycles are able to synchronize the circadian rhythms of embryos/larvae during the first days of development in the absence of lighting cues, which indicates that

thermocycles are a strong environmental signal for the ontogeny of the clock in fish (Lahiri et al., 2014). Likewise in tilapia in the present study, thermocycles enhanced the ontogeny of digestive circadian rhythms, which suggest an important role of thermocycles on fish circadian clock development, at least for freshwater species. In addition, it should be highlighted that tilapia in the wild is subjected to daily oscillations in temperature (Patterson and Wilson, 1995; Ndiwa et al., 2016). This species has evolved under these conditions, which might explain the impact of thermocycles on its physiology.

Finally, in the present study, thermocycles seemed to enhance the rhythmic expression of several genes, which in turn might have led to higher growth performance observed at 13 dpf. The mechanisms of action of thermocycles on the regulation of gene expression and how they can impact fish physiology remains unknown to date. Lahiri et al. (2005) suggested that, in the zebrafish, temperature cycles may act on the promoter of some genes, increasing or decreasing their expression through its action on an unknown enhancer element. In the case of clock genes and clock controlled genes, temperature would modify their phase and amplitude (Lahiri et al., 2005). In the present study, thermocycles led to a higher number of digestive factors displaying rhythmic variations, which might have occurred through their action at the gene promoter level. Strengthening rhythmic factors might have led to the differences observed in growth, *i.e.* by tuning the timing of feeding and digestive processes. However, with the available data, the link between these rhythms and growth cannot be confirmed so that further research is required to test this hypothesis.

5. Conclusions

In summary, our research shows that applying daily thermocycles during early development may improve the ontogeny and maturation of the daily rhythms of digestive enzymes and feeding regulatory hormones in tilapia. Larvae reared under thermocycles presented a higher growth at the last stage analyzed (13 dpf), which might be due to higher digestion rates and better food efficiency. Therefore, the use of thermocycles instead of constant temperatures in tilapia larviculture is interesting as it might help to improve rearing protocols, although understanding the mechanisms responsible for this response would require further research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735545.

Author contributions

AHES, JFLO, FJSV and PAPR designed the experiments; AHES, GA and YR performed the experiments; AHES, GA and JFLO analyzed data; JFLO, FJSV, RKL and PAPR contributed reagents/materials/analysis tools and others; AHES and JFLO wrote the paper with contribution from the rest of authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The present research was partially funded by projects CHRONOLIPOFISH (RTI2018-100678-A-I00), BLUESOLE (AGL2017-82582-C3-3-R) and CRONOFISH (RED2018-102487-T), granted by the Spanish Ministry of Science, Innovation and Universities, cofounded with FEDER, to JFLO and FJSV. The research was also partially funded by projects from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico)401117/2014-6, 308684/2017-6 and 308547/2018-7, granted to RKL and PAPR, and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). JFLO was funded by a "*Ramón y Cajal*" research fellowship granted by the Spanish MINECO (RYC-2016-20959).

References

Abramoff, M.D., Magalhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophoton. Int. 11, 36–42.

Almeida, A.P.G., Zardo, E.L., Toni, C., Behr, E.R., da Silva, L.P., Vieira, J.P., Loro, V.L., Baldisserotto, B., 2018. Composition of gastrointestinal content, protease and lipase activities in summer and winter of four freshwater siluriforms (Teleostei: Actinopterygii) with two different feeding habits. Zoologia 35, e13286.

Azaza, M.S., Legendre, M., Kraiem, M.M., Baras, E., 2010. Size-dependent effects of daily thermal fluctuations on the growth and size heterogeneity of Nile tilapia *Oreochromis niloticus*. J. Fish Biol. 76, 669–683.

Bennett, W.A., Beitinger, T.L., 1997. Temperature tolerance of the sheepshead minnow, *Cyprinodon variegatus*. Copeia 1, 77–87.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O., Sánchez-Vázquez, F.J., 2010. Effect of daily thermo-and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 306, 137–145.

Blanco-Vives, B., Vera, L.M., Ramos, J., Bayarri, M.J., Mañanós, E., Sánchez-Vázquez, F.J., 2011. Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in *Solea senegalensis*, kaup. J. Exp. Zool. A 315, 162–169.

Blier, P.U., Pelletier, D., Dutil, J.D., 1997. Does aerobic capacity set a limit on fish growth rate. Rev. Fish. Sci. 5, 323–340.

Boyd, C.E., 2004. Farm-level Issues in Aquaculture Certification: Tilapia. Report Commissioned by WWF-US. Auburn University, Alabama.

Brett, J.R., 1971. Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). Am. Zool. 11, 99–113.

Costa, L.S., Rosa, P.V., Fortes-Silva, R., Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2016a. Daily rhythms of the expression of genes from the somatotropic axis: the influence on tilapia (*Oreochromis niloticus*) of feeding and growth hormone administration at different times. Comp. Biochem. Physiol. C 181, 27–34.

Costa, L.S., Serrano, I., Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2016b. Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions. J. Comp. Physiol. B. 186, 775–785.

Crul, R.C.M., 1995. Limnology and hydrology of Lake Victoria, Comprehensive and Comparative Study of Great Lakes. UNESCO/IHP-IV Project M-5.1. UNESCO Publishing, Paris.

Cuesta, I.H., Lahiri, K., López-Olmeda, J.F., Loosli, F., Foulkes, N.S., Vallone, D., 2014. Differential maturation of rhythmic clock gene expression during early development in medaka (*Oryzias latipes*). Chronobiol. Int. 31, 468–478.

Culberson, S.D., Piedrahita, R.H., 1996. Aquaculture pond ecosystem model: temperature and dissolved oxygen prediction – mechanism and application. Ecol. Model. 89, 231–258.

de Alba, G., Mourad, N.M.N., Paredes, J.F., Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2019. Daily rhythms in the reproductive axis of Nile tilapia (*Oreochromis niloticus*): plasma steroids and gene expression in brain, pituitary, gonad and egg. Aquaculture 507, 313–321.

De Moura Pereira, M., Machado, E.M., Gisbert, E.R.E., 2019. Nile tilapia broodfish fed high-protein diets: digestive enzymes in eggs and larvae. Aquac. Res. 50, 2181–2190.

DeCoursey, P.J., 2004. The behavioral ecology and evolution of biological timing systems. In: Dunlap, J.C., Loros, J.J., DeCoursey, P.J. (Eds.), Chronobiology: Biological Timekeeping. Sinauer Associates, Sunderland, pp. 27–65.

Delgado, M.J., Cerdá-Reverter, J.M., Soengas, J.L., 2017. Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: involvement in the control of food intake. Front. Neurosci. 11, 354.

Di Rosa, V., Frigato, E., López-Olmeda, J.F., Sanchez-Vazquez, F.J., Bertolucci, C., 2015. The light wavelength affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae. PLoS One 10 (7), e0132235.

Durigon, E.G., Almeida, A.P.G., Jerônimo, G.T., Baldisserotto, B., Emerenciano, M.G.C., 2019. Digestive enzymes and parasitology of Nile tilapia juveniles raised in brackish biofloc water and fed with different digestible protein and digestible energy levels. Aquaculture 506, 35–41.

Fernandes, A.F.A., Alvarenga, É.R., Oliveira, D.A.A., Aleixo, C.G., Prado, S.A., Luz, R.K., Turra, E.M., 2013. Production of oocytes of Nile tilapia (*Oreochromis niloticus*) for in vitro fertilization via hormonal treatments. Reprod. Domest. Anim. 48, 1049–1055.

Fujimura, K., Okada, N., 2007. Development of the embryo, larva and early juvenile of Nile tilapia *Oreochromis niloticus* (Pisces: Cichlidae). Developmental staging system. Dev. Growth Differ. 49, 301–324.

Furné, M., Hidalgo, M.C., Lopez, A., Garcia-Gallego, M., MoraleS, A.E., Domezain, A., Domezaine, J., Sanz, A., 2005. Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*, a comparative study. Aquaculture 250, 391–398.

Gisbert, E., Morais, S., Moyano, F.J., 2013. Feeding and digestion. In: Qin, J.G. (Ed.), Larval Fish Aquaculture. Nova Science Publishers, Hauppauge, pp. 73–123.

Guerra-Santos, B., López-Olmeda, J.F., de Mattos, B.O., Baião, A.B., Pereira, D.S.P., Sánchez-Vázquez, F.J., Fortes-Silva, R., 2017. Synchronization to light and mealtime of daily rhythms of locomotor activity, plasma glucose and digestive enzymes in the Nile tilapia (*Oreochromis niloticus*). Comp. Biochem. Physiol. A 204, 40–47.

Jobling, M., 1995. Simple indices for the assessment of the influences of social environment on growth performance, exemplified by studies on Arctic charr. Aquac. Int. 3, 60–65.

Jomori, R.K., Carneiro, D.J., Malheiros, E.B., Portella, M.C., 2003. Growth and survival of pacu Piaractus mesopotamicus (Holmberg, 1887) juveniles reared in ponds or at different initial larviculture periods indoors. Aquaculture 221, 277–287.

Kamalam, B.S., Medale, F., Panserat, S., 2017. Utilisation of dietary carbohydrates in farmed fishes: new insights on influencing factors, biological limitations and future strategies. Aquaculture 467, 3–27.

Koven, W., Rojas-García, C., Finn, R., Tandler, A., Rønnestad, I., 2002. Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupea harengus*. Mar. Biol. 140, 1241–1247.

Lahiri, K., Vallone, D., Gondi, S.B., Santoriello, C., Dickmeis, T., Foulkes, N.S., 2005. Temperature regulates transcription in the zebrafish circadian clock. PLoS Biol. 3, e351.

Lahiri, K., Froehlich, N., Heyd, A., Foulkes, N.S., Vallone, D., 2014. Developmental stage-specific regulation of the circadian clock by temperature in zebrafish. Biomed. Res. Int. 2014, 930308.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408.

Lopes, Y.V., Henry-Silva, G.G., 2014. Effect of Nile tilapia culture on limnological variables in a reservoir of Rio Grande do Norte semiarid in a period of 24 hours. Bol. Inst. Pesca 40, 299–313.

López-Olmeda, J.F., 2017. Nonphotic entrainment in fish. Comp. Biochem. Physiol. A 203, 133–143.

López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2011. Thermal biology of zebrafish (*Danio rerio*). J. Therm. Biol. 36, 91–104.

López-Olmeda, J.F., López-García, I., Sánchez-Muros, M.J., Blanco-Vives, B., Aparicio, R., Sánchez-Vázquez, F.J., 2012. Daily rhythms of digestive physiology, metabolism and behaviour in the European eel (*Anguilla anguilla*). Aquac. Int. 20, 1085–1096.

Luz, R.K., Melillo Filho, R., Santos, A.E.H., Rodrigues, L.A., Takata, R., Alvarenga, É.R.D., Turra, E.M., 2012. Stocking density in the larviculture of Nile tilapia in saline water. R. Bras. Zootec. 41, 2385–2389.

Martín-Robles, Á.J., Whitmore, D., Pendon, C., Munoz-Cueto, J.A., 2013. Differential effects of transient constant light-dark conditions on daily rhythms of Period and Clock transcripts during Senegalese sole metamorphosis. Chronobiol. Int. 30, 699–710.

Mata-Sotres, J.A., Moyano, F.J., Martínez-Rodríguez, G., Yúfera, M., 2016. Daily rhythms of digestive enzyme activity and gene expression in gilthead seabream (*Sparus aurata*) during ontogeny. Comp. Biochem. Physiol. A 197, 43–51.

Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J., 2010. Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (*Sparus aurata*). Aquaculture 306, 315–321.

Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction? Comp. Biochem. Physiol. B 129, 243–249.

Narnaware, Y.K., Peyon, P.P., Lin, X., Peter, R.E., 2000. Regulation of food intake by neuropeptide Y in goldfish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, 1025–1034.

Navarro-Guillén, C., Moyano, F.J., Yúfera, M., 2015. Diel food intake and digestive enzyme production patterns in *Solea senegalensis* larvae. Aquaculture 435, 33–42.

Ndiwa, T.C., Nyingi, D.W., Claude, J., Agnèse, J.F., 2016. Morphological variations of wild populations of Nile tilapia (*Oreochromis niloticus*) living in extreme environmental conditions in the Kenyan Rift-Valley. Environ. Biol. Fish 99, 473–485.

Omondi, R., Kembenya, E., Nyamweya, C., Ouma, H., Machua, S.K., Ogari, Z., 2014. Recent limnological changes and their implication on fisheries in Lake Baringo, Kenya. J. Ecol. Nat. Environ. 6, 154–163.

Ospina-Álvarez, N., Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PLoS One 3 (7), e2837.

Pankhurst, N.W., King, H.R., 2010. Temperature and salmonid reproduction: implications for aquaculture. J. Fish Biol. 76, 69–85.

Patterson, G., Wilson, K.K., 1995. The influence of the diel climatic cycle on the depth-time distribution of phytoplankton and photosynthesis in a shallow equatorial lake (Lake Baringo, Kenya). Hydrobiologia 304, 1–8.

Peng, C., Peter, R.E., 1997. Neuroendocrine regulation of growth hormone secretion and growth in fish. Zool. Stud. 36, 79–89.

Perez-Casanova, J.C., Murray, H.M., Gallant, J.W., Ross, N.W., Douglas, S.E., Johnson, S.C., 2006. Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture 251, 377–401.

Qiang, J., He, J., Yang, H., Sun, Y.L., Tao, Y.F., Xu, P., Zhu, Z.X., 2017. Dietary lipid requirements of larval genetically improved farmed tilapia, *Oreochromis niloticus* (L.), and effects on growth performance, expression of digestive enzyme genes, and immune response. Aquac. Res. 48, 2827–2840.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquacult. 5, 59–98.

Sánchez-Vázquez, F.J., López-Olmeda, J.F., 2018. Environmental cycles and biological rhythms during early development. In: Yúfera, M. (Ed.), Emerging Issues in Fish Larvae Research. Springer International Publishing AG, Cham, pp. 37–50.

Schaefer, J., Ryan, A., 2006. Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. J. Fish Biol. 69, 722–734.

Silva, W.S., Costa, L.S., López-Olmeda, J.F., Costa, N.C.S., Santos, W.M., Ribeiro, P.A.P., Luz, R.K., 2019. Gene expression, enzyme activity and performance of Nile tilapia larvae fed with diets of different CP levels. Animal 13, 1376–1384.

Tengjaroenkul, B., Smith, B.J., Smith, S.A., Chatreewongsin, U., 2002. Ontogenic development of the intestinal enzymes of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture 211, 241–251.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40, e115.

Vera, L.M., De Pedro, N., Gomez-Milan, E., Delgado, M.J., Sanchez-Muros, M.J., Madrid, J.A., Sanchez-Vazquez, F.J., 2007. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiol. Behav. 90, 518–524.

Villamizar, N., Blanco-Vives, B., Migaud, H., Davie, A., Carboni, S., Sanchez-Vazquez, F.J., 2011. Effects of light during early larval development of some aquacultured teleosts: a review. Aquaculture 315, 86–94.

Villamizar, N., Ribas, L., Piferrer, F., Vera, L.M., Sánchez-Vázquez, F.J., 2012. Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. PLoS One 7 (12), e52153.

Volkoff, H., 2006. The role of neuropeptide Y, orexins, cocaine and amphetamine-related peptide, cholecystokinin, amylin and leptin in the regulation of feeding in fish. Comp. Biochem. Physiol. A 144, 325–331.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142, 3–19.

Yúfera, M., Moyano, F.J., Martínez-Rodríguez, G., 2018. The digestive function in developing fish larvae and fry. From molecular gene expression to enzymatic activity. In: Yúfera, M. (Ed.), Emerging Issues in Fish Larvae Research. Springer International Publishing AG, Cham, pp. 51–86.

Zeytin, S., Schulz, C., Ueberschär, B., 2016. Diurnal patterns of tryptic enzyme activity under different feeding regimes in gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 457, 85–90.

Zhang, C., Rahimnejad, S., Wang, Y.R., Lu, K., Song, K., Wang, L., Mai, K., 2018. Substituting fish meal with soybean meal in diets for Japanese sea bass (*Lateolabrax japonicus*): effects on growth, digestive enzymes activity, gut histology, and expression of gut inflammatory and transporter genes. Aquaculture 483, 173–182.

Ziv, L., Gothilf, Y., 2006. Circadian time-keeping during early stages of development. Proc. Natl. Acad. Sci. 103, 4146–4151.