UNIVERSIDADE FEDERAL DE MINAS GERAIS
Escola de Veterinária
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Walisson de Souza e Silva

Comportamento social de machos e suas implicações na reprodução e larvicultura do ciclídeo africano *Aulonocara nyassae*

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

Área de concentração: Produção Animal/Aquacultura

Prof. Orientador: Dr. Ronald Kennedy Luz

Coorientador: Dr. Lucas Pedro Gonçalves Junior

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FOLHA DE APROVAÇÃO

"Comportamento social de machos e suas implicações na reprodução e larvicultura do ciclídeo africano *Aulonocara nyassae*"

Walisson de Souza e Silva

Tese de Doutorado defendida e aprovada, no dia trinta de abril de dois mil e vinte e um pela Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Zootecnia da Universidade Federal de Minas Gerais, constituída pelos seguintes professores:

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pelos seguintes membros:

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"O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém" (Dalai Lama)

"Perguntam se você já se formou, se já se casou, se já tem filhos, como se a vida fosse uma lista de compras. Ninguém pergunta se você é feliz" (Criolo)

RESUMO

Os objetivos do presente trabalho foram avaliar o comportamento através da confecção de etogramas, a influência do *status* social dos machos na qualidade espermática, parâmetros hematológicos e bioquímica sérica, índices organossomáticos, desempenho zootécnico e reprodutivo de machos e fêmeas e, na larvicultura da progênie de *A. nyassae*. O experimento 1 foi dividido em: Fase 1 - estabelecimento dos *status* sociais entre os machos. Nesse período, os machos foram classificados de acordo com os *status* sociais: dominantes, subdominantes e submissos; Fase 2 - reprodução, foram selecionados quatro machos de cada *status* social e foram alocados com fêmeas e; Fase 3 - individualização dos machos (retirada das fêmeas). No experimento 2, na fase pré-experimental, quatro aquários, com três machos em cada, foram observados por 48 h e vídeos de 10 min foram gravados, duas vezes ao dia e foi gerado um etograma sobre definição *status* social. A fase experimental durou 66 dias, com um macho por aquário, cada um dos machos dos três *status* sociais foi alocado com três fêmeas, com quatro repetições. Os animais foram filmados por 10 min, duas vezes ao dia, por 5 dias, a cada 30 dias. Um etograma sobre comportamento reprodutivo de machos e fêmeas foi gerado. No experimento 1, na Fase 1, apenas um macho dominante foi detectado em um grupo de 10 animais. O peso final (PF) foi maior para os peixes dominantes. A concentração espermática, taxa de motilidade (MOT), deslocamento lateral (ALH), linearidade (LIN), retilinearidade (STR), oscilação (WOB) e batimento flagelar (BCF) não mostraram diferença estatística entre dominante e subdominantes. A velocidade da trajetória real (VCL), a velocidade da trajetória linear (VSL) e a velocidade da trajetória média (VAP) foram maiores para os peixes subdominantes. O tempo de motilidade foi maior para o macho dominante. Na fase 2, o desempenho dos machos, MOT e VAP foram maiores para os machos dominantes. ALH, LIN, STR, WOB e BCF foram maiores para machos subdominantes. Na fase 3, o desempenho, MOT, VCL, VSL, VAP e WOB foram superiores para machos dominantes e subdominantes. LIN e STR foram superiores para machos subdominantes. BCF foi superior para dominantes. No experimento 2, a locomoção foi mais abundante para machos subdominantes e submissos (P = 0.003). Para machos, o comportamento de corte foi mais abundante em machos dominantes e subdominantes e, em fêmeas com machos dominantes ($P < 0,05$). A locomoção foi maior em fêmeas com machos dominantes e subdominantes ($P < 0,05$). A sobrevivência de fêmeas com machos subdominantes foi menor ($P < 0,05$). O PF, ganho de peso médio (GPM), glicose (GLI), triglicerídeos (TG), os índices viscerossomático (IVS) e hepatossomático (IHS) para machos;

comprimento total médio (CTM), ganho de comprimento médio (GCM), fator de condição de Fulton (K), taxa de crescimento específico (TCE), hemoglobina (Hb), colesterol total (TC), GLI, PF e GPM para fêmeas e, para a progênie, a média de produção de ovos, taxa de eclosão, média de larvas produzidas, peso médio final das larvas e o comprimento médio final das larvas foram maiores para dominantes ($P < 0,05$). Em machos, a TC e TCE e, em fêmeas as proteínas plasmáticas totais (PPT), índice gonadossomático (IGS), índice de gordura intraperitoneal (IGIP), IHS, IVS e TG foram maiores para submissos ($P < 0,05$). Porém, GCM, IGS e IGIP, Hb e PPT foram maiores para machos subdominantes e submissos ($P < 0,05$). K foi maior para machos dominantes e submissos ($P < 0,05$). Portanto, os diferentes *status* sociais dos machos são determinantes para o desempenho, hemoglobina, bioquímica do sanguínea, qualidade espermática, capacidade reprodutiva de machos e fêmeas e, qualidade da progênie de *A. nyassae*.

Palavras-chave: Espermatozoide. Etograma. Peixe. Sangue. *Status* social

ABSTRACT

The aims of the present work were to evaluate behavior, evaluate the influence of male social status on sperm quality, hematological parameters and serum biochemistry, organosomatic indexes, growth and reproductive performance of males and females, and progeny larviculture of *A. nyassae*. Experiment 1 was divided into: Phase 1 - establishment of social status among males. During this period, males were classified according to social status: dominant, sub-dominant and submissive; Phase 2 - reproduction, four males of each social status were selected and were allocated with females and; Phase 3 - individualization of males (removal of females). In experiment 2, in the pre-experimental phase, four aquariums, with three males in each, were observed for 48 h and videos of 10 min were recorded twice a day and an ethogram was created about social status definition. The experimental phase lasted 66 days, with one male per aquarium, each of the males of the three social statuses was allocated with three females, with four replications. The animals were filmed for 10 min, twice a day, for 5 days, every 30 days. An ethogram on the reproductive behavior of males and females was generated. In Phase 1, only one dominant male was detected in a group of 10 animals. The final weight (FW) was higher for dominant fish. The sperm concentration, motility rate (MOT), Amplitude of lateral head movement (ALH), linearity (LIN), straightness (STR), wobble (WOB) and beat-cross frequency (BCF) did not show statistical difference between dominant and subdominant fish. Curvilinear velocity (VCL), Straight-line velocity (VSL) and Average path velocity (VAP) were higher for subdominant fish. Motility time was higher for the dominant male. In phase 2, the male performance was higher for dominant and subdominant. MOT and VAP were higher for dominant males. ALH, LIN, STR, WOB and BCF were higher for subdominant males. The social status of males did not influence the growth and reproductive variables of the females. In phase 3, the male performance was higher for dominant and subdominant fish. MOT, VCL, VSL, VAP and WOB were superior to dominant and subdominant males. LIN and STR were superior for subdominant males. BCF was superior to dominant. In experiment 2, locomotion was more abundant for subdominant and submissive males ($P < 0.05$). For males, courtship was greater in dominant and subdominant males and in females with dominant males ($P < 0.05$). Locomotion was higher in females with dominant and subdominant males ($P < 0.05$). The survival of females with subdominant males was lower ($P < 0.05$). FW, average weight gain (AWG), glucose (GLU), triglycerides (TG), viscerosomatic indexes (VSI) and hepatosomatic indexes (HIS) for males; average total length (ATL), average length gain ALG, Fulton's condiction factor (K), specific growth rate (SGR), hemoglobin (Hb), GLU, total cholesterol

(TC), FW and AWG for females and, for the progeny, the average egg production, hatching rate, average of produced larvae, final average weight of the larvae and the average length end of the larvae were higher for dominant (*P* < 0.05). In males, SGR and TC and, in female triglycerides (TG), total plasma protein (TPP), gonadosomatic index (GSI), intraperitoneal fat index (IPFI), HIS and VSI were higher for submissives (*P* < 0.05). However, ALG, GSI, IPFI, Hb and TPP were higher for subdominant and submissive males (*P* < 0.05). K was higher for dominant and submissive males (*P* < 0.05). Therefore, the social status of males influences the growth, hemoglobin, blood biochemistry, sperm quality, reproductive capacity of males and females and, progeny quality of *A. nyassae*.

Keywords: Blood. Ethogram. Fish. Social Status. Spermatozoid

LISTA DE FIGURAS

ARTIGO 1: Influence of social status on growth performance, reproductive success and sperm quality of the African cichlid *Aulonocara nyassae*

ARTIGO 2: Influence of the social status of male *Aulonocara nyassae* on behavior, growth, hematology, biochemical parameters, reproduction and larviculture

LISTA DE TABELAS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

°C	*Celsius degree* - Graus Celsius
Δt	Intervalo de tempo
µm	*Micrometre* – Micrômetro
%	*Percentage* – Porcentagem
ALH	*Amplitude of lateral head movement* – Amplitude de deslocamento lateral da cabeça
ANOVA	*Analysis of variance* – Análise de variância
APHA	*American Public Health Association*
ALG	*Average length gain*
ATL	*Average total length*
ATP	*Adenosine triphosphate* – Trifosfato de adenosina
AWG	*Average weight gain*
BCF	*Beat-cross frequency* – Frequência de batimento flagelar cruzado
$CaCO_3$	*Calcium carbonate* - Carbonato de cálcio
CASA	*Computer-Assisted Sperm Analysis* – Método computadorizado de análise de sêmen
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Comissão de Ética no Uso de Animais
cm	*Centimetre* – Centímetro
CTI	Comprimento total inicial
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CP	*Crude protein*
CTM	Comprimento total médio
CV	*Coefficient of variation* – coeficiente de variação
D	*Dominant* – Dominante
DAE	Dias após eclosão
DAH	*Days after hatching*
dL	*Decilitre* – decilitro
DNA	*Deoxyribonucleic acid* – Ácido desoxirribonucleico
DP	Desvio padrão
EV	Escola de Veterinária
FAO	*Food and Agriculture Organization* – Organização para a Alimentação e Agricultura

FTL	*Final total length*
FW	*Final weight*
G	*Gram* – Grama
GCM	Ganho de comprimento médio
Gli	Glicose
Glu	*Glucose*
GPM	Ganho de peso médio
GSI	*Gonadosomatic index*
Hb	*Hemoglobine* – hemoglobina
HSI	*Hepatosomatic index*
Hz	Hertz
IGS	Índice gonadossomático
IGIP	Índice de gordura intraperitoneal
IHS	Índice hepatossomático
IVS	Índice viscerossomático
IPFI	*Intraperitoneal fat index*
ITL	*Initial total length*
IW	*Initial weight*
K	*Fulton's condition factor* – Fator de condição de Fulton
kcal	*Kilocalorie* – quilocaloria
kg	*Kilogram* – quilograma
L	*Litre* – Litro
lx	Lux
LIN	*Linearity* – Linearidade
Laqua	Laboratório de Aquacultura
mg	*Milligram* – Miligrama
mm	*Millimetre* – Milímetro
MOT	*Motility* – Motilidade
mS	*Millisecond* – Milissegundo
N	*Number of animals* – número de animais
NT	*Non-territorial* – Não territorial
OD	*Oxidative phosphorylation* - Fosforilação oxidativa
PB	Proteína bruta

pH	*Hydrogenionic potential* – Potencial hidrogeniônico
PF	Peso final
PI	Peso inicial
PPT	Proteína plasmática total
S	*Submissive* - Submisso
SD	*Subdominant* – Subdominante
SGR	*Specific growth rate* – Taxa de crescimento específico
STR	*Straightness* – Retilinearidade
T	Territorial
TC	*Total cholesterol* – colesterol total
TG	*Triglycerides* – triglicerídeos
TPP	*Total plasmatic protein*
UFMG	Universidade Federal de Minas Gerais
VCL	*Curvilinear velocity* – Velocidade curvilínea
VSI	*Viscerosomatic index*
VSL	*Straight-line velocity* – Velocidade linear progressiva
VAP	*Average path velocity* – Velocidade média da trajetória
WOB	*Wobble* – Oscilação

SUMÁRIO

1. INTRODUÇÃO GERAL

Até o ano de 2030, a aquacultura será a principal fonte de obtenção de pescado, devido ao declínio da captura selvagem e estima-se que o haverá um crescimento de 32,2% na produção aquícola no Brasil (FAO, 2020). Segundo dados da FAO (2020), no ano de 2018 a produção da indústria pesqueira mundial, incluindo o cultivo de plantas aquáticas, atingiu 179 milhões de toneladas, com valor de venda estimado em US$ 401 bilhões, destes 82 milhões de toneladas e US$ 250 bilhões correspondem à aquacultura. A piscicultura brasileira alcançou a produção de 802.930 toneladas em 2020 com um crescimento de 5,93% sobre o ano anterior (Peixe BR, 2021).

Estudos sobre tecnologias de cultivo de peixes ornamentais vem se aprimorando (Faria et al., 2019). A produção de peixes ornamentais é uma indústria global e biodiversificada (Tlusty et al., 2013). Essa cultura envolve mais de 2500 espécies e a grande maioria corresponde a peixes de água doce (Dey, 2016). O mercado global desse ramo foi de US$ 4,2 bilhões em 2017, representando 10% do valor estimado pela aquicultura geral, com uma taxa de crescimento anual composta de mais de 7,85% (Techsci Research, 2019). Até meados de 2015, mais de 1 bilhão de peixes com destino ornamental foram comercializados internacionalmente a cada ano (Maceda-Veiga et al., 2016).

Dentre os peixes de interesse ornamental, se destaca a família dos ciclídeos, conhecidos pela variedade de cores entre as espécies, entre machos e fêmeas e, presença natural nas Américas do Sul, Central, México, África, Sudeste Asiático e Oriente Médio (Berra, 2001). Essa família é dividida em quatro classes: os ciclídeos da Índia (Etroplinae), Madagascar (Ptychochrominae), neotropicais (Cichlinae) e da África (Pseudocrenilabrinae) (Sparks e Smith, 2004). Esta família apresenta comportamento reprodutivo complexo, territorialismo e cuidado parental fáceis de serem visualizados (Keenleyside, 1991). Nesse grupo de peixes, a comunicação acústica, visual e química está associada a hierarquia social e influencia a reprodução, as relações co-específicas, refúgio e cuidado parental (Keller-Costa et al., 2016). Para a quantificação do comportamento, a confecção de etogramas com padrões de agressão e submissão de acordo com a espécie é uma ferramenta necessária (Martin and Bateson, 2007).

Dentre as espécies de ciclídeos, se notabiliza a *Aulonocara nyassae*, ciclídeo africano endêmico do Lago Malawi, apreciado pelo mercado ornamental pela coloração. Os machos, principalmente os dominantes, apresentam coloração azul-escuro, demonstrando o comportamento territorialista da espécie (Konings, 1995). Na maioria dos ciclídeos, os indivíduos considerados dominantes, apresentam melhores oportunidades reprodutivas e maior qualidade espermática, que, consequentemente, diminuem a capacidade reprodutiva dos subordinados (Fitzpatrick et al., 2006). Parâmetros sanguíneos e bioquímica sérica (Cyrino et al., 2000), índices organossomáticos e fator de condição de Fulton (Fazzio et al., 2019) estão diretamente ligados à respostas ao estresse e as relações de dominância podem os influenciar. Porém, as pesquisas com *A. nyassae* estão restritas à taxonomia e abrangência no ambiente natural.

Portanto, estudos sobre a influência dos *status* sociais sobre o desempenho reprodutivo e zootécnico, qualidade espermática, índices organossomáticos, parâmetros sanguíneos e bioquímica plasmática e etogramas espécie-específicos devem ser executados.

2. REVISÃO DE LITERATURA

2.1 A espécie *Aulonocara nyassae*

A ordem dos perciformes compreende cerca de um terço de todas as espécies de peixes e é a mais diversificada entre os vertebrados (Helfman et al., 2009). Dentre os perciformes, a família Cichlidae (ciclídeos) se sobressai pela grande quantidade de espécies registradas (aproximadamente 1900 espécies) (Weyl et al., 2010). Além disso, os ciclídeos estão presentes no Oriente Médio, América Central e do Sul, África e parte do Sudeste asiático (Berra, 2001).

Os grandes lagos africanos apresentam grande biodiversidade de espécies de peixes (Fryer e Iles, 1972). Dentre esses lagos, se destaca o Lago Malawi também conhecido como Lago Nyasa ou Lago Niassa, por estar entre os 10 maiores lagos do mundo e por possuir a maior quantidade de espécies de peixes registradas (Weyl et al., 2010). Os ciclídeos são a família de peixe com a maior abundância nesse lago, entre 800 e 1.000 espécies (Snoeks, 2004).

Segundo Konings (1995), os ciclídeos do gênero *Aulonocara* são nativos do Lago Malawi e apresentam diversas espécies com características morfológicas e comportamentais similares. Existem grupos de espécies que habitam naturalmente regiões mais rochosas e outras que habitam regiões mais arenosas do lago. Os ciclídeos encontrados nas porções arenosas se reproduzem nas partes mais rasas do lago e são capturados facilmente na natureza.

A espécie *Aulonocara nyassae* Regan 1922, conhecida mundialmente pelos nomes populares *african peacock*, *blue orchid*, *blue cichlid*, *emperor cichlid* e *peacock blue cichlid*, é um exemplar do grupo que se encontra nos locais arenosos. O sêmen dessa espécie é altamente concentrado e a motilidade média varia de 30 a 90% (Silva et al., 2021). As fêmeas desovam em ninhos em áreas arenosas construídos pelos machos.

A. nyassae é de interesse ornamental e está na lista de peixes exportados pela Tailândia, um dos maiores exportadores de peixes ornamentais (Monticini, 2010) e é citada como uma das espécies de peixe ornamental mais vendidas no estado do Ceará (Machado-Filho, 2018). Além disso, apresenta alto valor de mercado, sendo comercializada em sites de vendas peixes ornamentais entre US$3 a US$16,00 a unidade, com variação de 6 a 9 cm de comprimento, dependendo da região.

Desta forma, a coloração da espécie é de grande interesse comercial. Em pequenos grupos, apenas um macho apresenta coloração mais forte azul-escuro demonstrando o comportamento territorialista do *A. nyassae* (Konnings, 1995; Silva et al., 2021). Portanto, esses ciclídeos estabelecem *status* sociais.

2.2 Comportamento social e reprodutivo de ciclídeos

Ciclídeos têm sido amplamente estudados na área de controle social da reprodução, uma vez que exibem comportamentos sociais complexos, territorialidade e cuidado parental (Dewsbury, 1982).

O motivo pelo qual alguns animais da mesma espécie têm maior sucesso reprodutivo que outras, está relacionado à competição entre os machos e a escolha pelas fêmeas (Andersson, 1994). Segundo a teoria de investimento parental de Triver, o sexo que investe mais fortemente recursos energéticos na prole, à custa de potenciais filhos futuros, deve enfrentar a menor concorrência (Ahnesjö et al., 2008). Portanto, como as fêmeas apresentam maiores gastos energéticos com a prole, elas têm o direito de escolher o macho que vai oferecer melhor padrão genético e sobrevivência para a progênie (Candolin e Wong, 2008). Deste modo, a seleção intrassexual, geralmente mais forte entre machos, os selecionarão através da capacidade de combate e as fêmeas, por conseguinte, detectam as habilidades masculinas e escolhem o macho dominante (Candolin, 2000).

Os animais que vivem em grupos podem se estruturar em hierarquias, posições ou *status* sociais. O *status* de dominância em peixes pode ser influenciado por vários fatores, incluindo fase de desenvolvimento, capacidade reprodutiva, disponibilidade e qualidade do território, estado nutricional ou densidade e conformação do grupo (Maruska, 2014). Durante a fase reprodutiva, algumas espécies estabelecem uma hierarquia de dominância social que determina o acesso a recursos e a reprodução de indivíduos de posição hierárquica superior (Dewsbury, 1982).

Os machos dominantes ou territorialistas apresentam coloração mais intensa, defendem um território de desova e exibem comportamentos agressivos e de corte. Machos submissos ou não territoriais são de cor mais clara, não ocupam ou defendem territórios de desova e geralmente se agrupam com as fêmeas (Alonso et al., 2012). Quando um macho subordinado percebe uma oportunidade social de ascender ao *status* e se tornar dominante, a classe subdominante exibe comportamento territorial e reprodutivo (Alonso et al., 2012). Existem evidências de que esses machos ativam rapidamente o eixo hipotálamo-hipófise-gonadal e se tornam aptos para a reprodução (Maruska e Fernald, 2010).

Os ciclídeos africanos mais estudados na área de comportamento são *Astatotilapia burtoni* (Grosenick et al., 2007; Kustan et al., 2011; Maruska e Fernald, 2012; Fulmer et al., 2017), *Oreochromis niloticus* (Giaquinto e Volpato, 1997; Gonçalves-de-Freitas et al., 2008; Pfennig et al., 2012; Boscolo et al., 2018; Gonçalves-de-Freitas et al., 2019) e *Oreochromis mossambicus* (Barata et al., 2008).

Machos de *Astatotilapia burtoni* (Grosenick et al., 2007) podem estabelecer uma hierarquia entre outros machos a partir da observação de conflitos coespecíficos. Essa ascensão no *status* social pode ocorrer devido ao reconhecimento da força relativa de possíveis oponentes (Desjardins et al., 2012). Para esta mesma espécie, os machos não dominantes que ascendem na classificação social mostraram uma taxa de crescimento aumentada, enquanto os machos e animais territoriais que descem na classificação de *status* social apresentaram menor taxa de crescimento (Hofmann et al., 1999).

Em machos ciclídeos, geralmente, a capacidade reprodutiva está intimamente ligada ao *status* social (Fernald, 2002). A construção de ninhos e a manutenção dos ovos na boca são características do comportamento reprodutivo de diversas espécies de ciclídeos e a forma como é executada é espécie-específica (McKaye et al., 1990). Para o ciclídeo africano *O. niloticus*, machos constroem ninhos e defendem o território, as fêmeas escolhem um determinado macho como parceiro sexual, geralmente o macho dominante, em seguida ocorre o acasalamento, através de fertilização externa, fêmeas capturam os ovos e os incubam na boca até as larvas estarem prontas para a sobrevivência sem cuidado parental (Gonçalves-de-Freitas et al., 2019). Esses conflitos acontecem através da comunicação social entre os animais.

2.2.1 Comunicação social entre os peixes

A comunicação social em peixes acontece através de diversas modalidades sensoriais como a química, acústica e visual (Huertas et al., 2008; Huertas et al., 2014). A habilidade comunicativa é espécie-específica e em peixes de hábito noturno a comunicação visual é menos importante que a química e acústica (Keller-Costa et al., 2016). Contudo, em peixes de hábito diurno a visão é uma forma de interação importante (Giaquinto e Volpato, 1997).

A comunicação química é a maneira mais difundida de troca de informações entre os organismos, está relacionada ao *status* social e influencia a reprodução, as relações co-específicas, refúgio e cuidado parental em ciclídeos (Keller-Costa et al., 2016). Em peixes há evidências de seu envolvimento em interações de reconhecimento individual, familiar e territorialidade/dominância e ocorre através de sinais químicos detectados pelo sistema olfatório e eliminados através da urina (Giaquinto e Volpato, 1997; Barata et al., 2007; 2008).

Segundo Keller-Costa et al. (2016) os fatores químicos podem fornecer sinais de alarme que diminuem a agressão intraespecífica. Barata et al. (2007) demonstraram que o ciclídeo africano *O. mozambicus* armazena a urina e a usa para sinalizar sua posição dominante. Em *O. niloticus* os fatores químicos podem modular o reconhecimento co-específico, o que aumenta a estabilidade hierárquica e, portanto, diminui o confronto (Giaquinto e Volpato, 1997). Essa informação é endossada em um estudo com o ciclídeo neotropical *Pterophyllum scalare* na qual os sinais químicos são importantes para manter a estabilidade social dentro do grupo (Gauy et al., 2018). Contudo, os produtos químicos específicos e os mecanismos envolvidos nesse efeito ainda não são claros.

A comunicação acústica em peixes apresenta grande diversidade de mecanismos geradores de sons e produzem variedade de vocalizações importantes na resolução de conflitos, construção de ninhos, escolha de parceiros, territorialismo e defesa contra predadores (Ladich e Myrberg, 2006). Além disso, os peixes desenvolveram uma variedade de mecanismos de detecção de sons, que lhes permite ouvir sons inespecíficos e heteroespecíficos, incluindo a presença de predadores, presas, parceiros e rivais reprodutivos (Ladich e Schulz-Mirbach, 2016).

Os ciclídeos apresentam a capacidade de comunicação acústica e visual mais aprimorada, quando comparado a outras famílias de peixes pelo maior desenvolvimento das estruturas auditivas acessórias (Ladich e Schulz-Mirbach, 2016). A comunicação acústica geralmente vem seguida da comunicação visual (Myrberg, 2002). Na fase reprodutiva, o sucesso do acasalamento está altamente correlacionado ao status social sendo que as fêmeas podem usar pistas dos sons de cortejo do macho para avaliar a qualidade do parceiro, como diminuições na frequência de sons do dominante, podendo também estar relacionado ao aumento do tamanho do macho (Lobel e Mann, 1995). Em uma espécie de ciclídeo do Lago Malawi, o *Tramitichromis intermedius* foi verificado que a capacidade de um indivíduo produzir som e executar um comportamento de tremores ao mesmo tempo, pode ser um indicador da qualidade do parceiro pois, esse tipo de comportamento foi observado em machos submissos, mas esses indivíduos não produziram som e não fertilizaram ovócitos (Ripley e Lobela 2004). Portanto, para a definição dos *status* sociais, os animais comunicam entre si e a classificação do *status* social, comportamento social e reprodutivo pode ser observado através da confecção de etogramas.

2.3 Etograma

A etologia, do grego: *ethos*, “hábito” “maneira” e *logia*, “estudo” consiste no estudo da maneira como um animal ou um grupo de animais se comporta em determinado ambiente (Garcia, 1978). Esse ramo de estudo possui ferramentas para a determinação comportamental e uma delas é o etograma.

O etograma é um recurso de observação comportamental que permite a documentação e medição precisa dos comportamentos observados; portanto, sua construção é de fundamental importância no desenho de qualquer estudo etológico (Tinbergen, 1963). Esse recurso utiliza uma lista de padrões comportamentais da espécie estudada, geralmente relacionados à agressão e submissão (Martin e Bateson, 2007). Um etograma pode ser constituído de unidades, de acordo com observações qualitativas ou de acordo com a duração e frequência de cada unidade (Volpato e Gianquinto, 2001). É quantificado através do “DI”, que corresponde ao número de interações agressivas realizadas por um indivíduo dividido pelo número de interações agressivas do grupo (Gonçalves-de-Freitas et al., 2008) e deve ser elaborado de acordo com a espécie (Hick et al., 2014).

Hick et al. (2014) em um trabalho de comportamento de competição e socialidade em ciclídeos, elaboraram um etograma baseado sobre duas espécies de ciclídeos *Neolamprologus pulcher* e *Telmatochromis temporali*. Há postura agressiva sem ataque físico, na qual, peixe focal abaixa a cabeça e levanta a cauda na frente de seu oponente, exibição frontal, movimento de cabeça, batimento de cauda, pseudo-luta, agressão com ataques físicos evidentes, como a perseguição, cabeçadas, mordidas e confronto bucal. Em relação à postura submissa, a cabeça do peixe focal é direcionada para cima, às vezes inteiramente na vertical e a cauda está para baixo. Há exibição submissa, comportamento de gancho e fuga.

Em relação ao etograma durante a reprodução propriamente dita, foi descrito o comportamento do ciclídeo americano *Laetacara araguaiae* por Teresa e Gonçalves-Freitas (2011). Inicialmente, é relatado o comportamento de corte que dura até a desova. Nessa fase há exposição lateral, tremulação e batida de cauda. Esses movimentos ocorrem repetidamente e podem ser mostrados por um ou ambos os companheiros simultaneamente. Em seguida, há o comportamento de desova, na qual, o comportamento de machos e fêmeas são sincronizados. A fêmea faz movimentos circulares, pressiona a região ventral contra o substrato de areia e vibra a porção posterior do corpo ao entrar no ninho. Nesse momento, há liberação de ovócitos. Depois disso, o macho entra no ninho, realiza movimentos semelhantes aos da fêmea e há a liberação de sêmen. Posteriormente, machos e fêmeas revezam na entrada do ninho. A próxima fase é o cuidado parental, na qual, os reprodutores limpam ovos, aumentam o fluxo de água na desova e em seguida a fêmea os incuba na boca. Dias depois, há eclosão e as larvas nadam livremente.

Cada espécie apresenta particularidades no comportamento. O ciclídeo amazônico *Geophagus surinamensis*, por exemplo, exibe um tipo de comportamento agressivo não descrito para outras espécies de ciclídeos, o ataque bilateral, que consiste em sucessivos ataques em dois lados do oponente (Teresa e Gonçalves-Freitas, 2003). Brandão et al. (2018) e Gauy et al. (2019) descreveram que a espécie de ciclídeo americano *Cichlasoma paranaense* apresenta padrões de comportamento altamente agressivos na presença de peixes considerados oponentes. Essa agressividade se dá, majoritariamente por ataques laterais e confrontos frontais. Para *O. niloticus*, o ataque através da luta com a boca é a principal forma de agressão (Alvarenga e Volpato, 1995). No caso da espécie *Laetacara araguaiae*, a ameaça é a principal unidade comportamental, destacada pela ameaça lateral (Teresa e Gonçalves-Freitas, 2011). Portanto, existe uma grande diversidade de exibições comportamentais para os ciclídeos e elas podem influenciar o sucesso reprodutivo dos machos.

2.4 Qualidade espermática

O sucesso reprodutivo dos peixes é influenciado pela idade, *status* social, estado de saúde, qualidade de água, fatores estressores, alimentação e taxa de crescimento (Kowalski e Cejko, 2019). Em relação aos machos, a quantidade de espermatozoides (volume e concentração) e a qualidade (por exemplo, motilidade, pH do plasma seminal, composição e estabilidade da membrana e estabilidade do DNA) podem determinar a capacidade de fertilização e o sucesso reprodutivo (Ubilla et al., 2015). Os espermatozoides acessam, penetram o ovócito e quando há uma fertilização bem-sucedida, transmitem o genoma masculino (Cosson, 2019). Todo esse processo é influenciado pela motilidade dos espermatozoides.

A motilidade é um fator importante no sucesso da reprodução de peixes, pois, geralmente os espermatozoides de teleósteos permanecem móveis por um curto período após sua ativação (Melo e Godinho, 2006). A motilidade é induzida imediatamente após a liberação de espermatozoides do trato genital masculino no ambiente aquoso e depende da pressão osmótica, componentes iônicos e gasosos do meio externo e, em alguns casos, substâncias derivadas do ovo usadas para orientação espermática (Cosson, 2019). A duração da motilidade espermática tem importância para a fertilização, pois os espermatozoides precisam encontrar e penetrar a micrópila do ovócito antes desta fechar-se pela hidratação (Morales, 1986).

A avaliação do desempenho da motilidade de espermatozoides de peixes necessita, a princípio, de registros de vídeo de boa qualidade, de modo a medir a distância percorrida por cada cabeça de espermatozoides por um período correspondente (Cosson, 2019). Esses registros podem ser feitos através do método computadorizado de análise de sêmen (CASA).

O CASA, *Computer-Assisted Sperm Analysis*, lançado em 1975, é um sistema utilizado para visualizar, digitalizar e analisar imagens sucessivas, que fornece informações acuradas, precisas e significativas do movimento individual e de subpopulações de células espermáticas (Amann e Katz, 2004). Anteriormente, os pesquisadores só conseguiram estimar de forma subjetiva apenas a porcentagem de espermatozoides móveis e a duração total do movimento espermático (Gallego e Asturiano, 2018). O software reconhece as células e desenha para cada espermatozoide uma sequência completa do movimento para reconstituir sua trajetória, classificando-a conforme os padrões definidos como: móvel não progressivo, linear lento, linear rápido e imóvel (Mortimer, 2000).

Para a análise da motilidade propriamente dita, cada imagem captada pela câmera é convertida em imagem digital e o conjunto de imagens captadas em diversos campos de observação da amostra espermática em avaliação são automaticamente analisadas (Donald et al., 1988).

De acordo com Verstegen et al. (2002) os parâmetros reportados pelo CASA são:

- Velocidade curvilínea – *curvilinear velocity* (VCL; µm s^{-1}): é a velocidade da trajetória real do espermatozoide e, é sempre a maior das três velocidades e serve como elemento de cálculo para a linearidade;

- Velocidade linear progressiva – *straight-line velocity* (VSL; µm s^{-1}): é a velocidade média em função da linha reta estabelecida entre o primeiro e o último ponto da trajetória do espermatozoide e, é sempre a mais baixa das três velocidades;

- Velocidade média da trajetória - *average path velocity* (VAP; µm s^{-1}): é a velocidade da trajetória média do espermatozoide. Em casos em que a trajetória da cabeça espermática é muito regular e linear com pouco movimento lateral da cabeça, a VAP é quase a mesma que a VSL, porém, com trajetórias irregulares, não lineares ou onde existe maior movimento lateral, a VAP será maior que a VSL;

- Amplitude de deslocamento lateral da cabeça – *amplitude of lateral head movement* (ALH; µm): é a amplitude do deslocamento médio da cabeça do espermatozoide em sua trajetória real. A mensuração desse parâmetro está relacionada à capacidade de penetração na zona pelúcida do óvulo, assim, a ALH é um dos parâmetros que tem efeito sobre a fertilização;

- Frequência de batimento flagelar cruzado – *beat-cross frequency* (BCF; Hz): é o número de vezes que a cabeça do espermatozoide cruza a direção do movimento. Se existem mais batimentos/segundos que imagens/segundos, então, a BCF irá ser subestimada;

- Retilinearidade – *straightness* (STR; %): é a relação percentual entre VSL e VAP. Estima a proximidade do percurso da célula a uma linha reta;

- Oscilação – *wobble* (WOB = VAP/VCL; %): é a relação percentual entre VAP e VCL que mede a oscilação da trajetória real sobre a trajetória média. Este parâmetro pode ser útil para entender a eficiência do movimento médio para a frente e a progressão dividida pelos rendimentos do VAP.

- Linearidade - *linearity* (LIN; %): Relação percentual entre VSL e VCL, ou seja, é a porcentagem de células que tem index linear > 0.7, ângulo absoluto menor que 25° e ângulo algébrico menor que 3°. Quanto mais o espermatozoide se afasta da velocidade em linha reta, menor será sua linearidade.

Os valores de velocidade são determinados como percurso relevante percorrido em um período e são representados em µm/s, enquanto os valores de LIN e STR são determinados como raio dos valores de velocidade (Amann e Katz, 2004).

Além das avaliações da qualidade espermática, o sucesso reprodutivo e desempenho zootécnico são parâmetros que podem ajudar na compreensão dos *status* sociais de peixes.

2.5 Desempenho zootécnico, sucesso reprodutivo e qualidade da prole

Respostas de estresse em peixes geram consumo de reservas energéticas e perturbações na hosmeostase e prejudicam o desempenho zootécnico, a capacidade reprodutiva e a qualidade da prole em peixes (Urbinati e Carneiro, 2004). O estabelecimento do *status* social é uma situação de estresse e peixes considerados dominantes são sempre maiores que peixes submissos (Alonso et al., 2012).

As interações sociais influenciam na ingestão de alimento e apetite dos peixes (Houlihan et al., 2001; Eriegha e Ekokotu, 2017). Como consequência, animais submissos podem apresentar menor consumo de dieta e quando a alimentação não é adequada, as diferenças hierárquicas se tornam mais evidentes, há maior heterogeneidade do grupo e o desempenho é afetado negativamente (Houlihan et al., 2001). Porém, quando os animais com *status* social definido são separados, por alguma circunstância, peixes submissos e subdominantes podem apresentar taxa de crescimento igual ou superior aos dominantes (Alonso et al 2012). Esta situação foi relatada para os ciclídeos africanos *A. burtoni* (Hofmann et al., 1999), *Neolamprologus pulcher* (Riebli et al., 2011) e *A. nyassae* (Silva et al. 2021).

O baixo desempenho zootécnico das matrizes em relação às interações sociais, resulta em menor desenvolvimento gonadal, menor qualidade de ovos, menor sobrevivência e maior taxa de anormalidades larval que afetam toda a vida do animal (Izquierdo et al., 2001). Silva et al. (2021) relataram que *A. nyassae* dominantes e subdominantes apresentaram melhor qualidade espermática que peixes submissos. Contudo, a capacidade de fertilização e a qualidade da prole de cada *status* social não foi afetada. No referido estudo, durante a reprodução, os animais de cada hierarquia foram separados com fêmeas. Além disso, a coleta de sêmen de animais submissos só obteve sucesso quando estavam sozinhos nos tanques. Portanto, os autores concluíram que a capacidade reprodutiva de submissos é melhorada quando são individualizados durante a reprodução nessa espécie.

Além do desempenho zootécnico e reprodutivo e qualidade da prole, a avaliação dos parâmetros hematológicos e de bioquímica sérica também podem auxiliar o entendimento sobre *status* sociais de peixes.

2.6 Respostas fisiológicas e *status* social: hemoglobina, bioquímica sérica, índices organossomáticos e fator de condição (K)

O estabelecimento do *status* social em peixes é comumente associado à mudanças fisiológicas (Sloman et al., 2000). O estresse relacionado à organização hierárquica pode gerar custos metabólicos, alterações sanguíneas e bioquímicas que resultam em implicações negativas no sistema imunológico, crescimento e reprodução (Abbott et al., 2003). As respostas fisiológicas ao estresse social são mais evidentes em animais submissos, pois, apresentam maior gasto energéticos durante conflitos (Zayan, 1991). Porém, segundo Cutts et al. (1998), peixes dominantes são mais agressivos e podem apresentar maior custo metabólico em relação aos peixes submissos. Corrêa et al. (2003) observaram que, peixes dominantes e submissos apresentam a mesma intensidade de estresse durante o processo de estabelecimento do *status* social.

Os parâmetros hematológicos e bioquímicos indicam o estado de saúde dos peixes em resposta à nutrição, mudanças ambientais e doenças em condições de laboratório ou em sistemas de produção (Fazzio et al., 2019). Em adição, o comportamento e o habitat também podem influenciar os parâmetros hematológicos (Tavares-Dias e Moraes, 2007).

A hemoglobina é uma proteína relacionada à capacidade de transporte de oxigênio no organismo (Riggs, 1976). A concentração desta proteína no sangue é extremamente importante, pois, depende da condição do animal e a concentração de oxigênio dissolvido disponível no ambiente, ajudando na adaptação ambiental (Giardina et al., 2004; Perutz, 2004). Os níveis de hemoglobina podem diminuir, demonstrando um quadro anêmico, quando o peixe apresenta algum comprometimento na alimentação, nas brânquias, seja por conflitos, condições ambientais e doenças e, a capacidade de absorção de oxigênio é comprometida ou se elevam como uma forma de adaptação ao estresse que permite melhorar a capacidade de transporte de oxigênio (Misra et al., 2006).

As proteínas plasmáticas totais são formadas por albumina, fibrinogênio e globulinas e são responsáveis pelo transporte de hormônios (Saunders, 2013). A proteínas plasmáticas totais também estão relacionadas à demanda de energia em peixes através dos lipídeos (Javed e Usmani 2015), bem como a saúde e função hepática (Haschek et al. 2009).

As análises de glicose plasmática são amplamente utilizadas na pesquisa e são reconhecidamente bons indicadores da resposta ao estresse (Martínez-Porchas et al. 2009; Braithwaite e Ebbesson 2014). Níveis de glicose podem oscilar, promovendo alterações do balanço energético e, portanto, podem influenciar nos processos fisiológicos e reprodutivos (Shahjahan et al., 2014).

Os triglicerídeos são uma fonte de energia encontrada no fígado e nos músculos dos peixes teleósteos e são mobilizados durante a alta atividade metabólica (Lermen et al. 2004). Esse tipo de lipídeo se mobiliza em situações de estresse em peixes (Corrêa et al., 2003).

O colesterol também está diretamente relacionado ao estresse dos peixes (Mormède et al., 2007). Esse esteroide é um componente essencial das membranas celulares, precursor síntese de hormônios, prostaglandinas e atividade dos receptores de neurotransmissores (David and Michael, 2002). Em mamíferos, níveis baixos de colesterol estão associados a comportamento agressivo, pois, há diminuição da atividade da serotonina (Kaplan et al., 1997). Além disso, o colesterol é um precursor de hormônios esteroides e seus níveis séricos afetam diretamente as gônadas e, consequentemente, a reprodução (Lubzens et al., 2010). Portanto, em situações de estresse os níveis séricos de colesterol podem ser alterados e influenciar na fisiologia e reprodução dos peixes.

Corrêa et al. (2003), em um trabalho sobre relações de dominância em *Oreochromis niloticus*, relataram que machos mantidos em dupla, independente do *status* social, apresentaram um aumento na proteína plasmática, triglicerídeos e glicose quando comparado com animais isolados. Os autores sugerem que os conflitos de determinação de dominância causam mobilização de energia extra. Dessa forma, a avaliação de parâmetros hematológicos e bioquímicos vêm sendo utilizada como indicador de estresse.

Os índices organossomáticos também são parâmetros utilizados como indicadores do estado fisiológico dos peixes resultantes de respostas secundárias ao estresse (Tavares-Dias et al., 2007). Os índices viscerossomático (VSI), gonadossomático (GSI), hepatossomático (HSI) e gordura intraperitoneal (IGIP) consistem em um cálculo do peso das vísceras totais (incluindo a gordura), gônadas, fígado e gordura visceral, respectivamente. Variações na deposição de gordura influenciadas por fatores que alteram os parâmetros hematológicos e bioquímicos, como o estresse, podem gerar mudanças no volume do fígado, pelo acúmulo de glicogênio, que interferem nos índices VSI, GSI, HSI e VFSI (Cyrino et al., 2000). Gônadas mais volumosas podem ser indicativos de melhor capacidade reprodutiva (Adams et al., 1996). Porém, grande acúmulo de gordura visceral e volume exacerbado do fígado podem ser indicadores negativos quanto ao estado fisiológico dos peixes (Cyrino et al., 2000).

O fator de condição (fator de condição de Fulton - K) também é um parâmetro relacionado ao estresse (Froese, 2006), sendo calculado através da equação: $K = peso/comprimento^3$, na qual peixes mais pesados, com um determinado comprimento, indicam estar em melhores condições que animais de menor peso e com o mesmo comprimento (Froese, 2006).

3. OBJETIVOS

3.1 Objetivo geral

Avaliar a influência do *status* social de machos de *A. nyassae* no comportamento, desempenho reprodutivo e produtivo.

3.2 Objetivos específicos

- Avaliar a qualidade espermática de *A. nyassae* em três fases de cultivo nos diferentes *status* sociais através do método CASA em três fases de cultivo;
- Avaliar o desempenho zootécnico de machos de *A. nyassae* de diferentes *status* sociais em três fases de cultivo;
- Avaliar o desempenho zootécnico e reprodutivo de fêmeas de *A. nyassae* em reprodução com machos de diferentes *status* sociais;
- Avaliar a qualidade das desovas e larvicultura de *A. nyassae* de diferentes *status* sociais;
- Avaliar o desempenho zootécnico de machos de *A. nyassae* de diferentes *status* sociais durante 66 dias de reprodução;
- Avaliar a influência de machos de *A. nyassae* de diferentes *status* sociais sobre o desempenho zootécnico e reprodutivo de fêmeas durante 66 dias de reprodução;
- Avaliar a qualidade das desovas e larvicultura da prole oriundas de machos de *A. nyassae* de diferentes *status* sociais durante 66 dias de reprodução;
- Elaborar um etograma específico de machos de *A. nyassae* durante o estabelecimento de diferentes *status* sociais;
- Elaborar um etograma específico de *A. nyassae* durante a reprodução por 66 dias;
- Avaliar a hemoglobina, colesterol, triglicerídeos, glicose e proteína plasmática de machos de cada *status* social e fêmeas *A. nyassae*, após 66 dias de reprodução.

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5. ARTIGO 1

Influence of social status on growth performance, reproductive success and sperm quality of the African cichlid *Aulonocara nyassae*

Artigo publicado no periódico Applied Animal Behaviour Science (ANEXO A)

Silva, W.S., Gonçalves-Júnior, L.P., Henry, M.R.J.M., Ferreira, A.L., Torres, I.F.A., Neves, L.C., Ferreira, N.S., Luz, R.K., 2021. Influence of social status on growth performance, reproductive success and sperm quality of the African cichlid *Aulonocara nyassae*. Appl. Anim. Behav. Sci., 105292. https://doi.org/10.1016/j.applanim.2021.105292

Abstract

The status of social dominance may have consequences for the well-being of cichlids. These fish show territorial and aggressive behavior and are important for behavioral studies. The aim of the present study was to evaluate sperm variables of motility, growth performance and reproductive performance according to the social status of African cichlid *Aulonocara nyassae*. The experiment was divided into: Phase 1 - establishment of social statuses among males, 40 males of *A. nyassae* were kept in four tanks (42 L useful volume) with 10 animals in each tank for 30 days. During this period, the animals were classified according to social status as dominant, subdominant and submissive fish; Phase 2 – reproduction, one male of each social status (dominant, subdominant and submissive) per tank was selected, for a total of four males for each status and a total of 12 males. One male of each social status of each of original four tanks (total 12 males) was placed in a new tank with three females, for a total of 12 new tanks, and; Phase 3 - individualization of males (removal of females), the males were separated from their females and placed individually in 12 tanks. In Phase 1, only one dominant male was detected in a group of 10 animals, between 6 and 7 subdominant animals and, 2 and 3 submissive animals. The final weight (FW) was higher for dominant fish. The sperm concentration, motility rate (MOT), Amplitude of lateral head movement (ALH), linearity (LIN), straightness (STR), wobble (WOB) and beat-cross frequency (BCF) did not show statistical difference between dominant and subdominant fish. Curvilinear velocity (VCL), Straight-line velocity (VSL) and Average path velocity (VAP) were higher for subdominant fish. Motility time was higher for the dominant male. In phase 2, the male performance was higher for dominant and subdominant than submissive males. MOT and VAP were higher for dominant males. ALH, LIN, STR, WOB and BCF were higher for subdominant males. The social status of the males did not influence the growth and reproductive variables of the females. In phase 3, the male performance was higher for dominant and subdominant fish. MOT, VCL, VSL, VAP and WOB were superior to dominant and subdominant males. LIN and STR were superior for subdominant males. BCF was superior to dominant. Therefore, sperm quality and growth performance were influenced by changes in the social statuses.

Keywords: Cichlid, dominance, fish, submissive, reproduction, sperm quality

5.1 Introduction

Aggressive interactions are part of the natural behavior of several species of fish (Damsgård and Huntingford, 2012) and conflicts of interest are an important part of life in a social group (Buston, 2003). During the establishment of a social status, animals experience conflicts and go through a period of stress that may decrease when dominance relationships are defined (Haller and Wittenberger, 1988; Volpato and Fernandes, 1994). In such cases, the establishment of a social status may have consequences for animal health, survival and reproductive capacity (Sapolsky, 2005). Dominance status in fish may be influenced by age, body size, social and territorial composition and nutritional status (Maruska, 2014), and may have a direct influence on growth performance. According to Li and Brocksen (1977), there is a correlation between social status and growth of fish, by which animals considered dominant have higher growth rates than those considered submissive fish. Furthermore, environmental and social changes may have distinct effects on social status stability and upon individuals with differing ranks (Sneddon et al., 2006).

Some species establish a social status during the reproductive phase, which determines access to resources and reproduction for individuals at the highest level (Dewsbury, 1982). According to Baerends (1986), cichlid fish have revealed correlations between an individual color patterns and its developmental stage, sex, status, or behavior. Dominant or territorial males cichlids may be brilliantly colored, defend spawning territory and exhibit aggressive and courting behaviors (Maruska and Fernald, 2010; Alonso et al., 2012). Submissive or non-territorial males are lighter in color, do not occupy or defend spawning grounds and generally group with females (Alonso et al., 2012). A subordinate male may perceive an opportunity to ascend social status to become dominant and may exhibit territorial and reproductive behavior and quickly become able to reproduce, making it a subdominant male (Maruska and Fernald, 2010). All this social status establishment happens within a certain group, and as soon as animals are relocated in another environment, they tend to forget previous social experience (Hsu et al., 2006). In addition, in environments with higher densities individuals are more aggressive and have increased chances of forgetting previous social experience (Hsu et al., 2006).

Cichlids (Cichlidae) are a family of fish that exhibit complex reproductive behaviors, including territoriality and parental care, that are easy to view (Keenleyside, 1991). Acoustic, visual and chemical communication is associated with social status in these fish and influences reproduction, conspecific relationships, refuge and parental care (Keller-Costa et al., 2016). Males of the Lake Malawi cichlid, *Tramitichromis intermedius*, considered submissive fish were found not to produce sounds nor fertilize oocytes (Ripley, 2004). Therefore, social status influences the reproductive capacity of males. The various species of the cichlid genus *Aulonocara* are endemic to Lake Malawi and have similar morphological and behavioral characteristics. The species *Aulonocara nyassae* Regan 1922 is popularly known as African peacock, blue cichlid, emperor cichlid and blue peacock cichlid. When in small groups, only one male has a stronger dark-blue color, demonstrating its territorial behavior (Konings, 1995).

Social status can influence different reproductive strategies and even gonadal changes (Taborsky, 1994). For fish breeding, it is important to assess whether reproduction, including sperm quality, welfare and productivity may be influenced by social dominance. For example, sperm quality analysis for aquaculture purposes requires having quick and quantitative techniques available (Kime et al., 2001; Gallego and Asturiano, 2018). As Cichlid species are managed in captivity in various ways, e.g. for aquaculture and research, studies on how social behavior, including the establishment and maintenance of dominance relationships, influences growth and reproduction performance and sperm quality are necessary. The aim of this study was to evaluate sperm quality (CASA method), growth and reproductive performance of *Aulonocara nyassae* in relationship to social status.

5.2 Material and methods

The experiment was performed using 180-day-old adults of *Aulonocara nyassae* produced and cultivated at Laboratório de Aquacultura (Laqua), in six 42-L useful volume rectangular tanks at a density of 50 fish $tank^{-1}$, in a recirculating aquaculture system (RAS) and fed exclusively an extruded dry diet. Males were selected according to color. Females do not have a bluish color. The experiment was divided into three phases.

5.2.1 Phase 1- Evaluation of growth and sperm of *A. nyassae* during social status division

For Phase 1, 40 males of *A. nyassae* (7.56±2.71 g, 7.18±0.66 cm) were kept in four tanks (42 L useful volume in RAS) with 10 males in each tank for 30 days. During this period, the males were classified according to social status as dominant, subdominant and submissive fish (Beeching, 1995; Maruska and Fernald, 2010). The social status (and thus the social status of individual fish) in each tank was visually defined daily by observing according to colour and territorial behavior of fish for one minute. At the end of 30 days, the number of animals of each social status per tank was determined by observation. Dominant animals were those that showed a high degree of aggression, territoriality, faster swimming, dark blue in colour throughout the body and close to the operculum, animals presented dark yellow colour (Fig.s 1A, 1B). Submissive animals were those that always exhibited escape behavior, sought shelter or often looked for regions closer to the water surface of the tanks had more pronounced opercular movements, less marked blue color and no yellow color in the opercular region (Fig. 1A, 1B). Subdominant animals were those that showed dominance over submissive males but were submissive to dominant males, had a light blue color throughout the entire body, a light yellow color in the opercular region, felt cornered by dominant individuals and more aggressive towards submissive animals (Fig.s 1A, 1B). One animal of each social status (dominant, subdominant and submissive males) in each tank was subsequently anesthetized in Eugenol (50 mg L^{-1}) for semen collection. Semen was collected through massage of the urogenital region with the aid of a pipette, transferred to Eppendorf tubes and cooled at -20 °C. There were two hours between the first semen collection and the last computer-assisted sperm analysis (CASA).

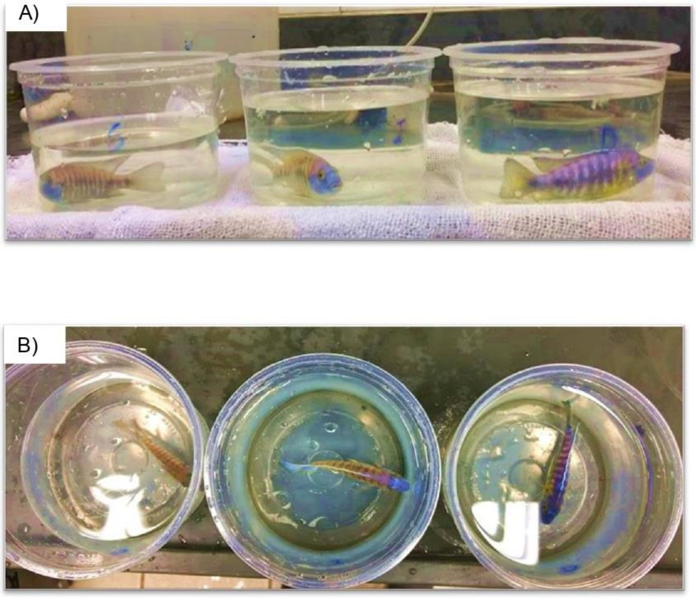

Fig. 1. Images of males of *A. nyassae* in different social status classified during the experimental period. Side view of submissive, subdominant and dominant males, from left to right (A). Top view of submissive, subdominant and dominant males, from left to right (B).

5.2.2 <u>Phase 2- Growth, reproduction and sperm quality of *A. nyassae* after social status division</u>

At the end of Phase 1, one male of each social status (dominant, subdominant and submissive) per tank was selected, for a total of four males for each status and a total of 12 males. The males had initial weights and lengths of, respectively, 13.81±1.34 g and 9.68±0.54 cm for dominant fish, 12.27±1.30 g and 9.35±0.30 cm for subdominant fish and 7.49±1.04 g and 8.35±0.37 cm for submissive fish. Thirty-six females (5.57±1.13 g, 7.24±0.45 cm) produced in the laboratory were also selected. One male of each social status of each of the original four tanks (total 12 males) was placed in a new tank (42 L useful volume in RAS) with three females, for a total of 12 new tanks.

The experimental design was completely randomized with three treatments (social status: dominant, subdominant and submissive males) and four repetitions each. The semen of males was collected after 30 days as described in Phase 1. Females were observed daily, and when spawning was detected, larvae were collected on the fifth day after observation (hatching). The larvae were kept in a thermostatic bath at 28.3±0.3 °C in containers containing 1 L of water at a density of 10 L^{-1} larvae and fed three times a day with a commercial mash diet containing 46% crude protein (CP). Twice a day, 50% of the water volume was changed in order to maintain water quality. After 30 days, the larvae were counted, weighed and measured. These data were used to determine spawning frequency, average number of eggs produced, number of larvae produced and survival at 30 days.

5.2.3 Phase 3- Growth and sperm quality of *A. nyassae* of different social statuses after female separation

At the end of Phase 2, four males of each social status were separated from their females and placed individually in 12 tanks (42 L useful volume in RAS) and fed as described in Phase 1. The males had initial weights and lengths of, respectively, 15.33±1.30 g and 10.35±0.37 cm for dominant fish, 15.05±0.59 g and 10.20±0.48 cm for subdominant fish and 9.22±1.12 g and 8.35±0.37 cm for submissive fish. The semen of the males was collected after 30 days as described in Phase 1. The experimental design was completely randomized with three treatments (dominant, subdominant and submissive males) and four replications per treatment.

5.2.4 Experimental protocols

Fish were kept in 42-L tanks (84 cm x 25 cm x 20 cm in height) in RAS for all three phases. Each tank had a 30 cm long and 10 cm diameter pipe as shelter. The animals were fed three times a day (08:00, 12:00 and 16:00 h) until apparent satiety with an extruded commercial diet (Acqualine Supra®) of 1.2–1.8 mm thickness, containing 46% CP, 8% ether extract and 3600 kcal kg^{-1} of digestible energy. The photoperiod was kept at 12 h light:12 h dark and water quality variables were measured three days a week prior to the first daily feeding. Dissolved oxygen was measured using a POL-69 Politerm oximeter (Politerm®, São Paulo, Brazil) and was maintained at 5.59±0.52 mg L^{-1}, while pH, temperature, salinity and conductivity were measured using a HI98129 Hanna Instruments Combo PH\EC\TDS\Temperature meter (Hanna Instruments Brazil®) and were kept at 7.21±0.15, 28.38±0.39 °C, 0.20±0.01 g L^{-1} and 0.41 ± 0.02 mS cm^{-1}, respectively. Total ammonia and nitrite were measured by means of commercial kits (Alcon®) and were kept at 0.21±0.07 mg L^{-1} and 0.1±0.05 mg L^{-1}, respectively. Alkalinity was measured by American Public Health Association (APHA) (1998 Protocol) and was kept at 104.44±6.45mg L^{-1} of $CaCO_3$.

At the end of each phase, all animals used for semen collection were measured and weighed with the aid of Starrett 799 series digital caliper (150 mm capacity, ± 0.02 mm accuracy; Starrett®) and a AS5500 Marte digital scale (capacity 5 kg, ±0.01 g accuracy; Marte Científica®), respectively.

Data of weight, length and feed consumption were used to calculate:

- Final Weight (g) (FW);
- Average Weight Gain (g) (AWG) = Final Weight (FW) - Initial Weight (IW);
- Specific Growth Rate (% day^{-1}) (SGR) = SGR = 100 (ln Pf - ln Pi)/Δt

where Pi is initial weight; Pf is final weight and Δt is duration of days between samplings.

- Average Total Length (cm) (ATL);
- Average Length Gain (g) (ALG) = Final Length (FL) - Initial Length (IL).

5.2.5 Computer-Assisted Sperm Analysis (CASA)

For the analysis of semen motility and kinetics, refrigerated samples were transported to Laboratório de Reprodução em Criopreservação (Crioreprolab) located at Departamento de Clínica e Cirurgia Veterinárias of Escola de Veterinária of UFMG. Analyses were performed using the CASA system (Sperm Class Analyzer model - SCA®, version 4.0, MICROPTIC®, Barcelona, Spain), with the following variables for system calibration (setup): 40 microns^2, particle area; four samples per treatment in triplicate, with a 20 second interval between images. For the analysis of each sample, 5 μL of semen and 200 μL of distilled water, used as an activating solution, were directly deposited on a slide previously heated to 27 °C, and covered by a coverslip (24x24 mm) under a trilocular microscope previously focused with a 40x objective. Five homogeneous fields were captured, with a minimum of 500 sperm cells in each field of each sample. The following CASA kinetic variables were measured, according to the methodology proposed by Farrell et al. (1998): motility time (s) = time in which the sperm remain mobile; sperm concentration (M mL^{-1}) = amount of sperm in a given sample volume, motility rate (MOT; %) = percentage estimate of mobile sperm; flagellar beat (BCF; Hz) = number of times that the head of the sperm crosses the direction of the movement; linearity (LIN; LIN = VSL/VCL,%) = Percentage relationship between VSL and VCL, that is, it is the percentage of cell that has a linear index >0.7, absolute angle less than 25°C and algebraic angle less than 3°; speed of the real trajectory (VCL; μms^{-1}) = Speed of the actual trajectory of sperm; linear path velocity (VSL; μms^{-1}) = average speed as a function of the straight line established between the first and the last point of the sperm trajectory; average path velocity (VAP; μms^{-1}) = speed of the average sperm trajectory; straightness (STR; STR = VSL/VAP; %) = ratio between VSL and VAP. Estimates the proximity of the cell path to a straight line; oscillation (WOB; WOB = VAP/VCL; %) = ratio between VAP and VCL which measures the oscillation of the actual trajectory of the average trajectory and; lateral displacement amplitude (ALH; μm) = Amplitude of the average displacement of the head of the sperm in its real trajectory. The measurement of this parameter is related to the ability to penetrate the egg's pellucid zone.

5.2.6 Statistical analyses

Data of growth performance (final weight, average weight gain, specific growth rate, average total length, average length gain), sperm quality (motility time, sperm concentration, motility rate, flagellar beat, linearity, speed of the real trajectory linear path velocity, average path velocity, straightness, oscillation and lateral displacement amplitude) and reproductive variables (total average spawning frequency, average number of eggs produced, average number of larvae, average larval survival), were analyzed by the Shapiro-Wilk normality test and the Levene test for homoscedasticity using Minitab statistical software (Minitab® version 17) and all data showed normality and homoscedasticity. Sperm quality data were analyzed in four repetitions (N=4) and each semen sample was analyzed in triplicate. All data subsequently submitted to ANOVA to compare the distribution of the groups tested in independent samples. In which, in the null hypothesis (H0) = there is no difference between social status according to the variables tested and, in the alternative hypothesis (H1) = There is at least one social status with difference in the tested variables. For all variables (growth performance, sperm quality and reproductive variables), the fixed sources of variation were social status, the tank of origin of the animals and the error. As there are three observed social statuses, when the "F" test for ANOVA treatments (analysis of variance) was significant in a certain variable, the multiple-mean comparison test called Tukey's test was used, at the level of 5% probability ($P < 0.05$). Responses were expressed as means ± standard deviation (SD).

5.2.7 Ethical approval

All methods used in this study were approved by the Commission of Ethics in the Use of Animals of UFMG (Comissão de Ética no Uso de Animais da UFMG, Protocolo CEUA 329/2019). The aggressive behavior analyzed never generated any observable physical damage and the fish were not subjected to any invasive method.

5.3 Results

5.3.1 Phase 1- Evaluation of growth and sperm of *A. nyassae* after social status division

In Phase 1, production performance as measures through final weight (FW; Fig. 2A), average weight gain (AWG; Fig. 2B), specific growth rate (SGR; Fig. 2C), average total length (ATL; Fig. 2D) and average length gain (ALG; Fig. 2E) were higher for dominant and subdominant animals and lower for submissive animals (P = 0.001, P = 0.001, P = 0.001, P = 0.004 and P = 0.004, respectively).

Only one dominant fish was detected for each one of four groups of 10 animals, while six and seven subdominant animals were detected in two tanks and, two submissive animals were detected in two tanks and three submissive animals were detected in two tanks. There was no mortality during the experimental period. None of the submissive males released seminal fluid with massage of the celomatic cavity. Therefore, the analyzes were made only for fish of dominant and submissive social status. Sperm concentration (Fig. 3B), ALH (Fig. 3G), LIN (Fig. 3H), STR (Fig. 3I), WOB (Fig. 3J) and BCF (Fig. 3K) did not differ significantly between dominant and subdominant males (P = 0.911, P = 0.657, P = 0.198, P = 0.420, P = 0.248 and P = 0.376, respectively). Values for VCL (Fig. 3D), VSL (Fig. 3E) and VAP (Fig. 3F) were highest for subdominant animals (P = 0.037, P = 0.039 and P = 0.048, respectively), while motility time (Fig. 3A) and MOT (Fig. 3C) were high for dominant males (P = 0.006 and P = 0.01, respectively).

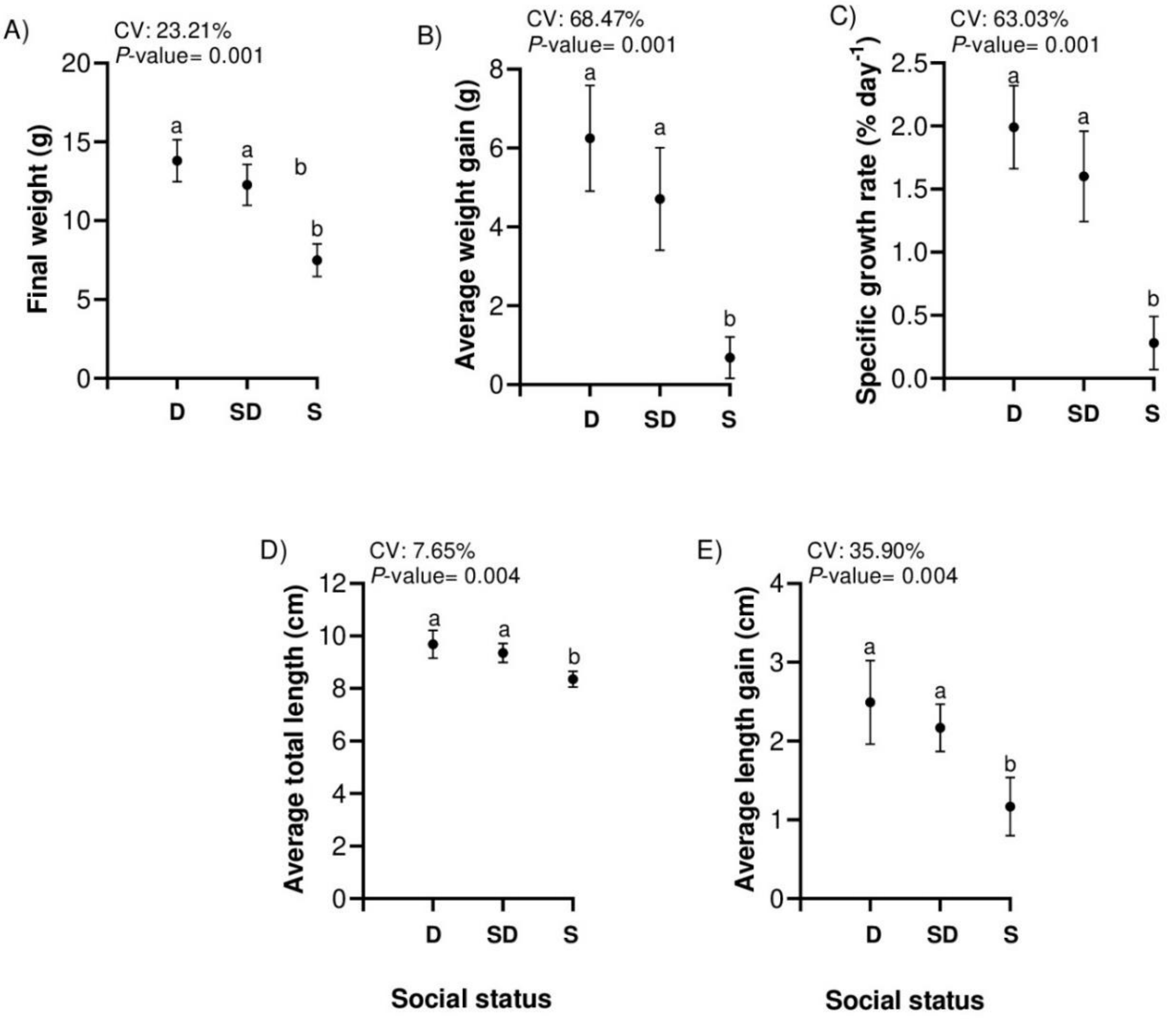

Fig. 2. Final weight (A), average weight gain (B), specific growth rate (C), average total length (D) and average length gain (E) of males of *A. nyassae* in different individualized social status in the tanks (phase 1), after 30 days of isolation.

Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation.

D: dominant; SD: subdominant; S: submissive.

CV: Coefficient of variation

N = 4 animals per treatment

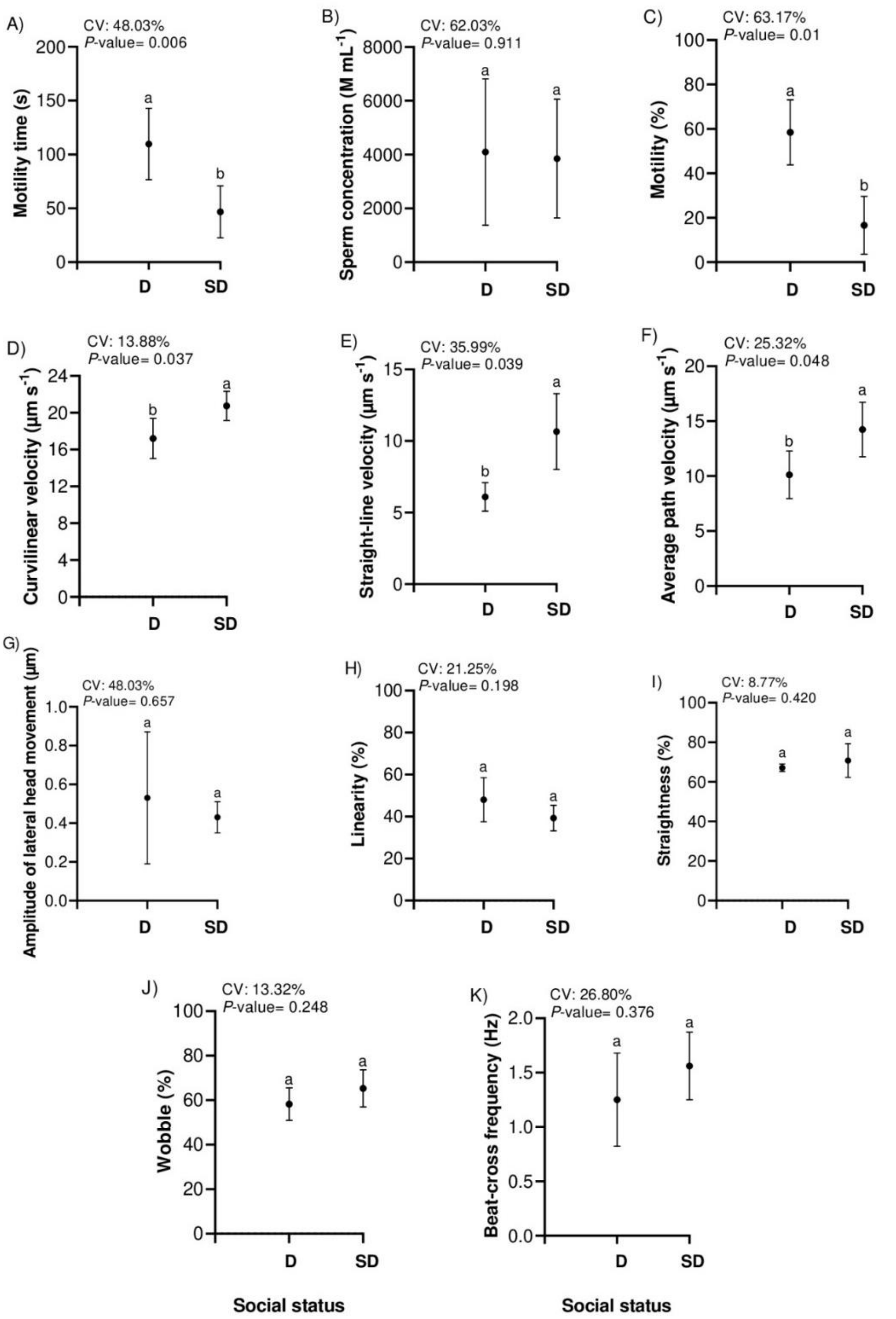

Fig. 3. Variation in motility time (A), sperm concentration (B), motility rate (C), curvilinear velocity (D), linear path velocity (E), average path velocity (F), Amplitude of lateral head movement (G), linearity (H), straightness (I), wobble (J) e flagellar beat (K) of males of *A. nyassae* in 20 seconds after activation at the end of Phase 1, by the CASA method, in relation to three social status, after 30 days of interaction.

Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation.

D: dominant; SD: subdominant; S: submissive.

CV: Coefficient of variation

N = 4 animals per treatment in triplicate

5.3.2 Phase 2- Growth, reproduction and sperm quality of *A. nyassae* after social status division

In Phase 2, previously dominant and subdominant males had higher initial weight (IW; Fig. 4A), final weight (FW; Fig. 4B), initial total length (ITL; Fig. 4E) and average total length (ATL; Fig. 4F) than did submissive males (P = 0.001, P = 0.001, P = 0.004 and P = 0.001, respectively) (Fig. 4). Subdominant males had high values for average weight gain (AWG; Fig. 4C) and average length gain (ALG; Fig. 4G), while previously dominant and submissive males had low values (P = 0.012 and P = 0.006, respectively). However, specific growth rate (SGR; Fig. 4D) was higher for previously subdominant males, intermediate for previously submissive males and lower for previously dominant males (P = 0.009).

All growth variables evaluated in females were similar, regardless of the social status of the males present in the tank: final weight (FW) = 6.67±0.76 g (P = 0.07), average weight gain (AWG) = 1.37 ± 0.75 g (P = 0.320), specific growth rate (SGR) = 0.80 ± 0.45 % day^{-1} (P = 0.230), average total length (ATL) = 7.92±0.42 cm (P = 0.08) and average length gain (ALG) = 0.69 ± 0.44 cm (P = 0.158).

None of the variables related to the evaluated eggs and larvae were influenced by social status. Total average spawning frequency per female in each social status was 1.11 ± 0.18 (P = 0.100); the average number of eggs produced was 24.44 ± 6.13 (P = 0.09); the average number of larvae produced at 30 days was 12.22 ± 3.46 (P = 0.150); and average larval survival at 30 days was 58.3 ± 15.5% (P = 0.202).

Sperm variables were similar to those for Phase 1, with none of the previously submissive males releasing seminal fluid with massage of the celomatic cavity. Therefore, the analyzes were made only for fish of dominant and submissive social status. Motility time (Fig. 5A), sperm concentration (Fig. 5B), VCL (Fig. 5D) and VSL (Fig. 5E) did not differ significantly between previously dominant and previously subdominant males (P = 0.350, P = 0.448, P = 0.107 and P = 0.087, respectively), while VAP (Fig. 5F), ALH (Fig. 5G), STR (Fig. 5I), WOB (Fig. 5J) and BCF (Fig. 5K) were higher for previously subdominant males (P = 0.448, P = 0.350, P = 0.107 and P = 0.087, respectively). The highest values for MOT (Fig. 5C) and LIN (Fig. 5H) were for previously dominant males P = 0.003 and P = 0.001, respectively).

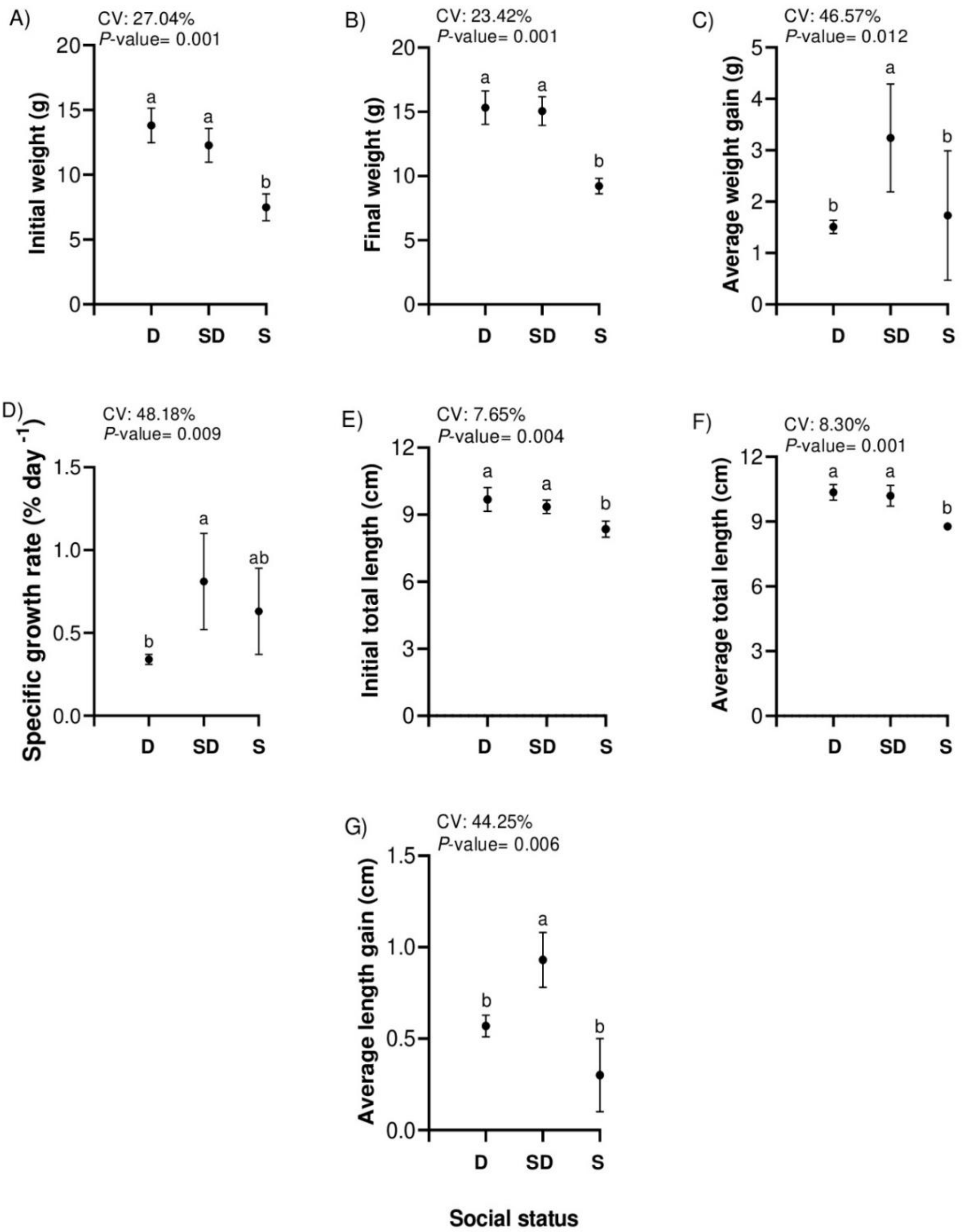

Fig. 4. Initial weight (A), Final weight (B), average weight gain (C), specific growth rate (D), initial total length (E), average total length (F) and average length gain (G) of males of *A. nyassae* in different individualized social status in the tanks (phase 2), after 30 days of isolation. Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation.
D: dominant; SD: subdominant; S: submissive.
CV: Coefficient of variation
N = 4 animals per treatment

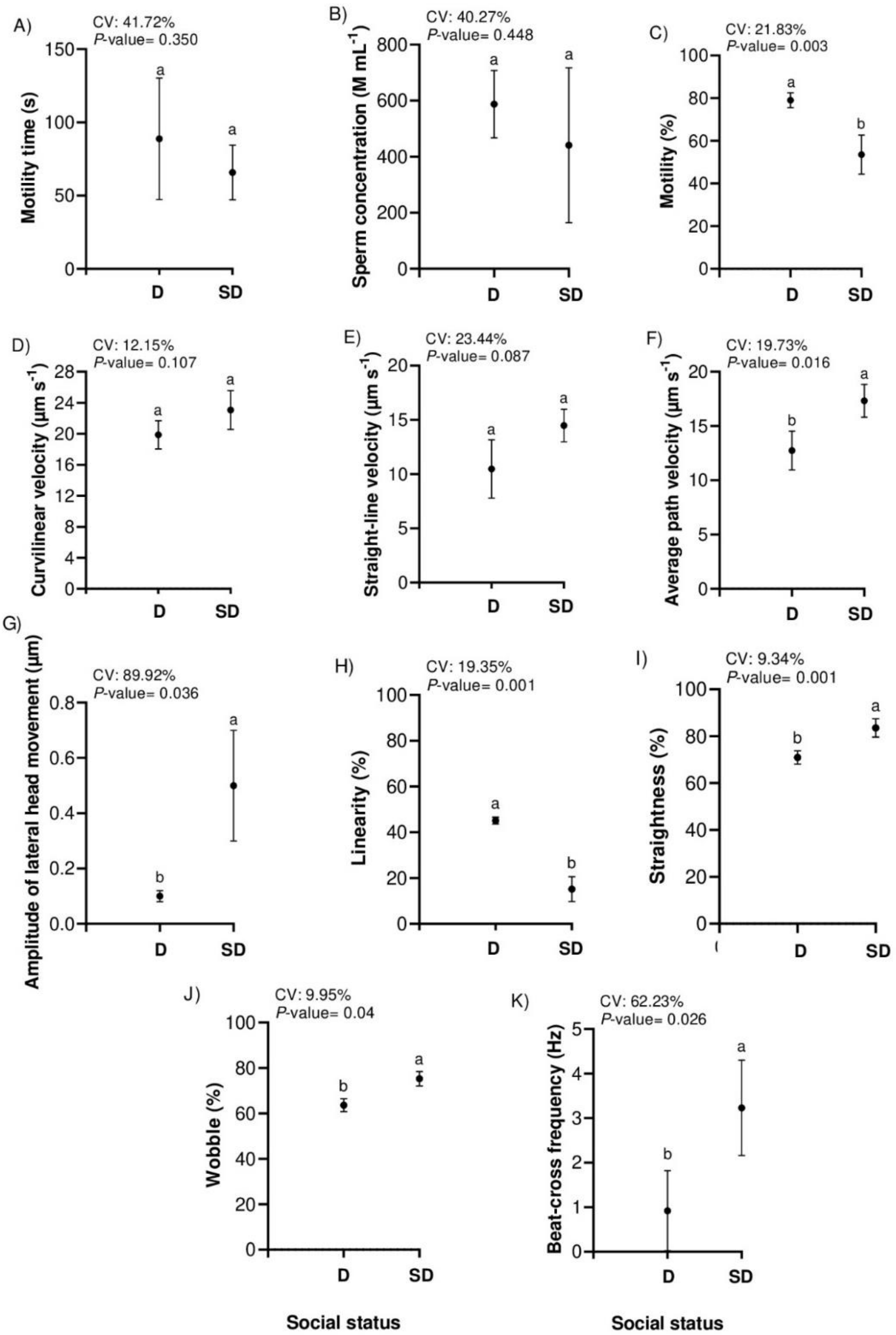

Fig. 5. Variation in motility time (A), sperm concentration (B), motility rate (C), curvilinear velocity (D), linear path velocity (E), average path velocity (F), Amplitude of lateral head movement (G), linearity (H), straightness (I), wobble (J) e flagellar beat (K) of males of *A. nyassae* in 20 seconds after activation at the end of Phase 2, by the CASA method, in relation to three social statuses, after 30 days of interaction.

Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation

D: dominant; SD: subdominant; S: submissive.

CV: Coefficient of variation

N = 4 animals per treatment in triplicate

5.3.3 Phase 3- Growth and sperm quality of *A. nyassae* of different social statuses after female separation

At Phase 3, previously dominant and previously subdominant males had higher initial weight (IW; Fig. 6A), final weight (FW; Fig. 6B), initial total length (ITL; Fig. 6E) and average total length (ATL; Fig. 6F) than previously submissive males (P = 0.001, P = 0.001, P = 0.001 and P = 0.004, respectively) (Fig. 6), while average weight gain (AWG; Fig. 6C), specific growth rate (SGR; Fig. 6D) and average length gain (ALG; Fig. 6G) were similar for all tested social statuses levels (P = 0.362, P = 0.170 and P = 0.156, respectively).

Unlike phases 1 and 2, sufficient seminal fluid was obtained in Phase 3 for analyses of all three social statuses, including submissive males. Motility time (Fig. 7A), sperm concentration (Fig. 7B) and ALH (Fig. 6G) were not influenced by social status (P = 0.354, P = 0.314 and P = 0.241, respectively). However, MOT (Fig. 7C), VCL (Fig. 7D), VSL (Fig. 7E), VAP (Fig.7F) and WOB (Fig. 7J) were higher for previously dominant and previously subdominant males and lower for previously submissive males (P = 0.020, P = 0.007, P = 0.05, P = 0.023 and P = 0.03, respectively); LIN (Fig. 7H) and STR (Fig. 7I) were higher for previously subdominant males, intermediate for previously dominant males and lower for previously submissive (P = 0.026 and P = 0.048, respectively); and BCF (Fig. 7K) was higher for previously dominant males and lower for previously subdominant and previously submissive males (P = 0.029).

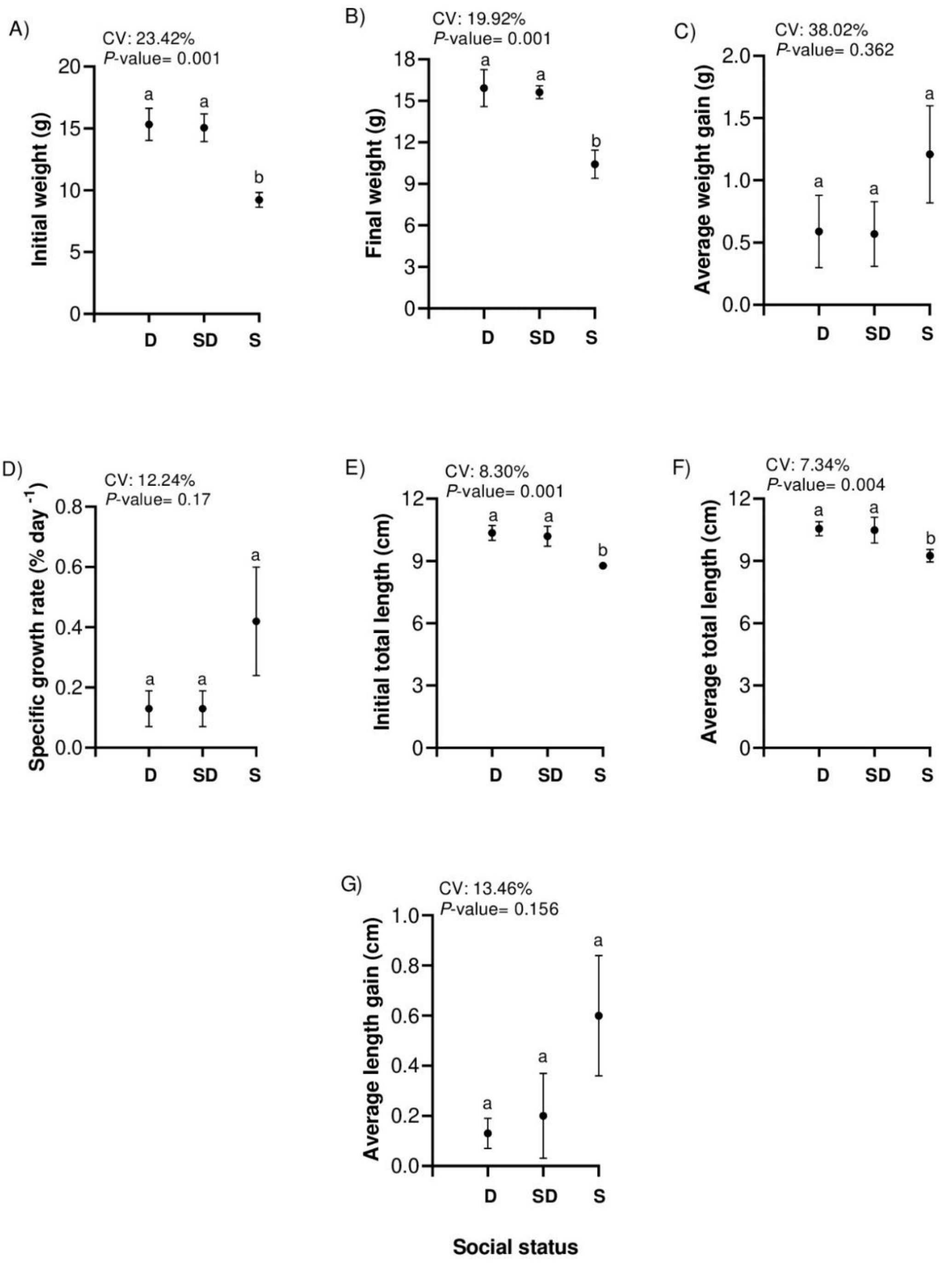

Fig. 6. Initial weight (A), Final weight (B), average weight gain (C), specific growth rate (D), initial total length (E), average total length (F) and average length gain (G) of males of *A. nyassae* in different individualized social statuses in the tanks (phase 3), after 30 days of isolation.

Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation.

D: dominant; SD: subdominant; S: submissive.

CV: Coefficient of variation

N = 4 animals per treatment

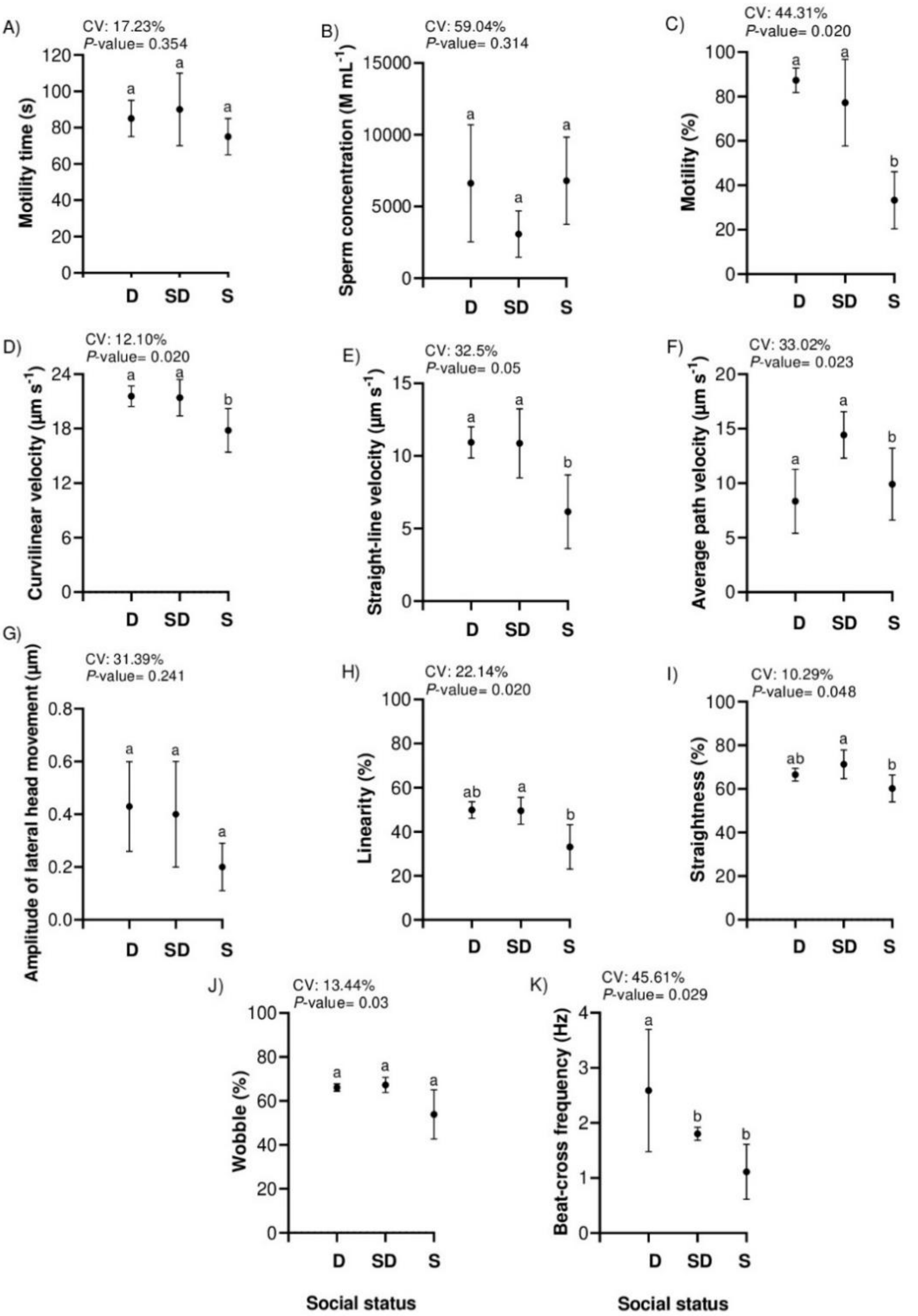

Fig. 7. Variation in motility time (A), sperm concentration (B), motility rate (C), curvilinear velocity (D), linear path velocity (E), average path velocity (F), Amplitude of lateral head movement (G), linearity (H), straightness (I), wobble (J) e flagellar beat (K) of males of *A. nyassae* in 20 seconds after activation, at the end of Phase 3, by the CASA method, in relation to three social statuses, after 30 days of isolation.

Different letters indicate significant differences ($P < 0.05$) by the Tukey test. Data are expressed as mean ± standard deviation.

D: dominant; SD: subdominant; S: submissive.

CV: Coefficient of variation

N = 4 animals per treatment in triplicate

5.4 Discussion

The different social statuses influenced sperm quality and growth performance of reproducing males of *A. nyassae*.

In the groups of 10 males, only one fish was ever considered dominant, between 60 and 70% were subdominant fish and 20 to 30% submissive fish. Similar to the present study, Maruska and Fernald (2013) reported that dominant (territorial) males of the African cichlid *Astatotilapia burtoni* also represented a small percentage of the population (10 to 30%), while subdominant and submissive males represented the majority (70 to 90%). According to these same authors, fish of these latter social statuses are lighter in color, have no territories and swim away from aggressive dominant males. In addition, dominant males of *A. burtoni* are bolder in color, differ in behavioral and hormonal profiles and may reversibly change color, suggesting that they may use color change as a flexible behavioral strategy (Korzan et al., 2008). Such color patterns were also documented in the present study.

Social status may directly affect growth performance, as seen in the present study when dominant and subdominant males had greater growth in weight and length than did subordinate males. This indicates the effect of dominant males on the growth of subdominant and submissive males and that *A. nyassae* is a territorial rather than gregarious species. According to Alonso et al. (2012), the dominant or ascending fish in the social status of the American cichlid, *C. dimerus*, is always larger than submissive fish. The results of the present study were also similar to those reported for *A. burtoni*, in which subdominant males that ascended the social status had increased growth rates (Hofmann et al., 1999). The same was verified for the African cichlid *Neolamprologus pulcher* (Riebli et al., 2011). This regulatory mechanism for the growth of individuals in social ascension occurs because more energy is spent on growth and less on territorial defense, aggressive interactions and gonadal growth, and, thus, fish are more likely to achieve reproductive opportunity in the future when they ascend the social status (Alonso et al., 2012).

The previous social status of males did not influence the growth performance of females during reproduction (Phase 2). According to Alonso et al. (2012), males of *C. dimerus* are more aggressive than females, and dominant individuals are more aggressive than submissive individuals. According to these same authors, the behavior of females of this species is influenced more by motivation and personality than by social status. However, in the present study, the reproductive capacity of females was not affected.

Regarding sperm variables, dominant and subdominant individuals had greater motility according to a collection of four animals per treatment in triplicates. Previously subdominant individuals showed improvement in sperm variables in phases 2 and 3, when they were isolated from males considered dominant in Phase 1. However, sperm concentration remained similar between subdominant and dominant males. Submissive individuals did not have sufficient sperm volume for analysis in phases 1 and 2; however, they fertilized oocytes and had reproductive capacity (offspring) similar to animals of the social statuses considered superior. Sufficient sperm volume was determined only in Phase 3. The most important variables in the evaluation of semen quality are MOT and sperm velocity. The parameter MOT is directly correlated with oocyte fertilization (Viveiros et al., 2010). According to Kowalski and Cejko (2019), MOT is species specific. In general, high quality sperm should show sperm motility above 80%, however, MOT may decrease by 9% every hour between collection and analysis (Chao et al., 1987). For *Cyprinus carpio*, MOT was found to vary from 29% to 98.4% (Ceiko et al., 2018) while for the African cichlid, *Oreochromis niloticus*, MOT has been reported to vary from 55.3 to 90.8% (Sarmento et al., 2017) and from 77.9% to 81.0% (Mataveli et al., 2007). However, in the present study, MOT changed among statuses, on average from 58.45 to 87.31% for dominant individuals, from 16.65 to 77.2% for subdominant individuals and from 33.33 to 46.23% for submissive individuals (Phase 3 only). Therefore, social status influences sperm quality.

In the present study, variables of speed and movement (VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF) varied according to social status. Dominant and, mainly, subdominant males had better results for the tested speed variables. In general, the values found for speed variables for *A. nyassae* in the present study are lower than those reported for other species in the literature, such as *Carassius carassius* (Cejko et al., 2013a; Cejko et al., 2013b; Cejko et al., 2015) and *C. carpio* (Kowalski et al., 2014; Cejko and Kucharczyk, 2015, Cejko et al., 2018), for example. This variation may be due to species-specific variation, in addition to the difficulty in collecting semen, as they are small animals with small amounts of sperm.

For teleost fish species with external fertilization, physiological quality of sperm differs among competing males due to differences in investment in gametes and their production (Vladić and Järvi, 2001). Sperm of these fish need to travel only short distances to fertilize oocytes, and they do this by performing circular movements around them until they penetrate the micropyle (Rurangwa et al., 2004). Therefore, curved movement is important for these species. All of this movement is made possible by mitochondria, which are responsible for most cellular energy production (Copeland, 2002). This organelle plays an important role in the kinetic movements of sperm because ATP is produced in this structure through oxidative phosphorylation (OD). During spermiogenesis, mitochondria move towards the developing flagellum scourge, while others are progressively released by the sperm cell (De Martino et al., 1979). A reduction in the amount of this source of energy would, therefore, decrease the ability of sperm to swim (Marques and Godinho, 2004). Thus, individuals considered submissive fish were observed to have poor growth performance and, in general, poor sperm quality, as the sperm had less physiological capacity to move; however, offspring quality for submissive individuals was not affected. Despite low sperm volume, these males managed to fertilize females. Perhaps in an environment with males of higher social status, such submissive individuals would not have this same capacity for fertilization. According to Sneddon et al. (2011), dominant animals have less physiological stress than subdominant and submissive animals. This stress may be a factor responsible for higher sperm quality in animals of higher social status.

Changes related to sperm quality and growth performance in phases 2 and 3 compared to phase 1 may be related to changes in social conformation. Animals tend to forget previous social experience when they are submitted to another group and this new experience generates new conflicts (Hsu et al., 2006). In addition, fish called submissive in a previous group, can increase growth rate when they are placed individually in tanks and become more reproductively effective.

5.5 Conclusion

Sperm quality and growth performance of *A. nyassae* are influenced by social status. Dominant and subdominant males had a better sperm quality and performance than submissive males. However, social status of the male did not influence the reproductive capacity and growth performance of females. Previous subdominant and submissive males experienced improved growth performance in relation to specific growth rate when separated from dominant males. Therefore, our study suggests that changes in group composition of *A. nyassae* may influence growth and sperm quality. These findings may have implications related to the management of cichlid species under human care, as in aquaculture and research laboratories, and also in improving the maintenance conditions of these animals in order to obtain better well-being according to their behavior. Future experimentation should assess the impact of environmental variation that may have a major impact on behavior.

5.6 Acknowledgements

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6. ARTIGO 2

Influence of the social status of male *Aulonocara nyassae* on behavior, growth, hematology, biochemical parameters, reproduction and larviculture

Artigo publicado no periódico Aquaculture (ANEXO B)

Silva, W.S., Gonçalves-Júnior, L.P., Ferreira, A.L., Neves, L.C., Ferreira, N.S., Luz, R.K., 2021. Influence of the social status of male *Aulonocara nyassae* on behavior, growth, hematology, biochemical parameters, reproduction and larviculture. Aquaculture 738197. https://doi.org/10.1016/j.aquaculture.2022.738197

Abstract

The aim of the present study was to evaluate the influence of social status of *Aulonocara nyassae* males on behavior, growth and reproductive performance, hematological parameters and male and female biochemicals and the success of offspring larviculture. For the pre-experimental phase, four aquariums with three males each were observed for 48 h and videos of 10 min, twice a day, were recorded and an ethogram was generated on the definition of social status, classified according to aggressive behavior, territorialism and coloration as dominant, subdominant and submissive. The experimental phase lasted 66 days. One male was stocked per aquarium, from each of the three social statuses, with three females and four replicates. The animals were filmed for 10 min, twice a day, for 5 days, every 30 days. An ethogram on the reproductive behavior of males and females was generated. Locomotion was more frequent in subdominant and submissive males and in females with dominant and subdominant males ($P < 0.05$). Courtship behavior was more frequent in dominant and subdominant males and in females from aquariums with dominant males ($P < 0.05$). The survival of females with subdominant males was lower ($P < 0.05$). Final weight (FW), average weight gain (AWG), glucose (GLU), triglycerides (TG), viscerosomatic (VSI) and hepatosomatic (HSI) indices for males; final mean total length (FTL), mean length gain (MLG), hemoglobin (Hb), specific growth rate (SGR), Fulton condition factor (K), total cholesterol (TC), FW, AWG, GLU and for females and for progeny, average of egg production, hatching rate, average of larvae produced, mean final larvae weight and mean final larvae length were higher for dominants. In males, SGR and FTL, in females, total plasma protein (TPP), gonadosomatic index (GSI), intraperitoneal fat index (IPFI), TG, HSI and VSI were higher for submissive ($P < 0.05$). However, the mean length gain (MLG), GSI and IPFI, Hb and TPP were higher for subdominant and submissive males ($P < 0.05$). K was higher for dominant and submissive males ($P < 0.05$). Therefore, the social status of males influences growth performance, hematological, biochemical parameters and reproductive capacity of males and females of *A. nyassae*.

Keywords: blue orchid, cichlid, dominance, ethogram, Lake Malawi, progeny

6.1 Introduction

Aulonocara nyassae Regan 1922, is an African cichlid species inhabiting sandy regions of Lake Malawi known by the popular names african peacock, blue orchid, blue cichlid, emperor cichlid, and peacock blue cichlid (Konings, 1995). When in small groups, only one male exhibits stronger dark-blue coloration, demonstrating the territorial behavior of the species (Konings, 1995; Silva et al., 2021).

Studies on behavior and social status in fish have mainly focused on the African cichlids *Astatotilapia burtoni* (Grosenick et al., 2007; Kustan et al., 2011; Maruska and Fernald, 2012; Fulmer et al., 2017), *Oreochromis niloticus* (Giaquinto and Volpato, 1997; Gonçalves-de-Freitas et al., 2008; Pfennig et al., 2012; Boscolo et al., 2018) and *Oreochromis mossambicus* (Barata et al., 2008), with reproductive capacity being closely linked to social status (Fernald, 2002).

Males of some cichlid species can be divided into three main social statuses: dominant, subdominant, and submissive (Alonso et al., 2012; Fulmer et al., 2017 and Silva et al., 2021). Dominant or territorial males are more offensive and territorial and have strong and pronounced colors (Alonso et al., 2012). Subdominant or non-territorial males are more defensive and exhibit more opaque colors (Alonso et al., 2012). Subdominant males are less aggressive than dominant and more territorial than submissive males (Fulmer et al., 2017), and may ascend in social status and exhibit territorial behavior according to the environmental situation (Alonso et al., 2012).

Such behavior patterns related to social groups can be assessed by ethogram construction. An ethogram consists of observations that accurately document and measure observed behaviors (Tinbergen, 1963). This resource is based on patterns of aggression, submission, and reproduction of a given species (Martin and Bateson, 2007). Therefore, cichlids possess a wide diversity of behavioral displays, which may affect their development (Alonso et al., 2012).

Dominant fish are generally larger and submissive fish show a higher growth rate when they are separated from dominant fish (Hofmann et al., 1999; Riebli et al., 2011; Silva et al. 2021). Low growth performance of matrices related to social interactions results in lower gonadal development, lower egg quality, lower survival, and a higher rate of larval abnormalities, which affect the entire life of the animal (Izquierdo et al., 2001). Furthermore, dominant and subdominant fish have been found to have better sperm quality than submissive fish, but with no difference in fertilization capacity and progeny quality among social statuses (Silva et al., 2021).

Social interactions may also cause physiological changes (Abbott et al., 2003). Dominant and submissive fish exhibit the same intensity of stress when establishing social status (Corrêa et al., 2003). Serum hematological and biochemical parameters may indicate the health status of fish in response to nutrition, environmental changes, and disease under laboratory conditions or in production systems (Fazzio et al., 2019).

Organosomatic indexes are also considered complementary parameters used as indicators of the physiological state of fish (Tavares-Dias et al., 2007). The deposition of fat, influenced by factors that alter hematological and biochemical parameters, such as stress, leads to changes in the volume of the liver and gonads and may interfere with viscerosomatic (VSI), gonadosomatic (GSI), hepatosomatic (HSI) and intraperitoneal fat (IPFI) indices (Cyrino et al., 2000).

Thus, the aim of the present study was to evaluate the influence of the social status of *A. nyassae* males on the reproductive behavior, growth performance and reproductive success of males and females. In addition, to evaluate this influence on the hematological and biochemical parameters of males and females, as well as on the larviculture of the progeny.

6.2 Material and methods

An experiment was carried out at the Laboratório de Aquacultura (Laqua) of the Escola de Veterinária of the Universidade Federal de Minas Gerais (UFMG), Brazil. All methods used in this study were approved by the Comissão de Ética no Uso de Animais da UFMG (CEUA Protocol 16/2021). Adult *Aulonocara nyassae* at 18 months post-hatching, produced at Laqua, were used. The animals were fed three times a day (08:00, 12:00 and 16:00 h) until apparent satiation with a commercial extruded diet (Acqualine Supra® Alisul alimentos S.A, Anápolis, Goiás, Brazil) with a thickness of 1.2–1.8 mm and containing 46% crude protein (CP), 8% ethereal extract (EE) and 3600 kcal kg^{-1} of digestible energy (DE). Larvae were fed with the same diet although crumbled.

6.2.1 Animals and experimental conditions

The pre-experimental phase involved dividing males according to social status, while in the experimental phase the divided males were placed in aquariums with females for breeding.

6.2.1.1 Pre-experimental phase

A total of 96 adult *A. nyassae* were kept in four 48-L aquaria (24 per aquarium) in a recirculating aquaculture system (RAS). The fish were separated by sex according to secondary characteristics, specifically blue body coloration in males and yellow-gray body coloration in females. Twelve males (mean initial weight of 9.62±1.50 g, mean total length of 8.77±0.62 cm), were initially allocated, in a completely randomized design (CRD), among four 48-L aquaria (three per aquarium) in a RAS with a mechanical and biological filter and supplemental aeration.

The animals were observed for 24 h and video recorded for 10 min at 9:00 h and 15:00 h (Adapted from Fulmer et al., 2017) to obtain data regarding dominance behavior. Recordings were made using a Moto G4 Plus Camera (16 megapixel 4608x3456 pixels, Full HD, Motorola©, Schaumburg, Illinois, USA), positioned in front of the aquaria. Fish were allowed to acclimate to the presence of the observer for 10 min before each initial recording. During this 10-min period, the animals were classified according to social status as dominant, subdominant, or submissive (Maruska and Fernald, 2010). According to Silva et al (2021), dominant *A. nyassae* are those that show a high degree of aggression and territorialism and a visibly more pronounced blue color. Submissive individuals are those that isolate themselves in the corners of aquaria and show less pronounced blue coloration. Subdominant individuals are those that demonstrated dominance over submissive individuals but are submissive to dominant individuals. The animals considered dominant were remove and placed individually in 48-L aquaria while the two remaining animals in each aquarium were kept together for another 24 h with 10 min video recordings at 09:00 and 15:00 h to establish subdominant and submissive animals.

Because there is no established observation protocol for aggressive behavior for *A. nyassae*, observations followed the method described by Altmann (1974) and were classified as:

- Made to order, with free choice;
- Casual;
- Used for initial approach.

Interactions between males in each tank were observed and quantified to acquire data on social status. The data were then used to generate an ethogram (Table 1) using Solomon Coder software (version beta 19.08.02) and the data stored for later comparison of the behavior of the animals.

Table 1. Ethogram of *A. nyassae* males during the establishment of social status for two days.

Observation	Behavior description
Threat of aggression behaviors	
Exhibition	
Front and perpendicular display	The focal fish approaches another fish and extends its operculum and lower jaw.
Lateral display	The focal fish quickly approaches the opponent and then quickly swims away.
Permanence in the shelter	The focal fish goes to the shelter and defends the shelter from the other fish.
Physical aggression	
Lateral attack	The focal fish touches the opponent laterally.
Chasing	The focal fish chases the opponent.
Head-butting	The focal fish uses its head to attack the opponent.
Biting	The focal fish swims towards the other and chases it out of its mouth.
Cornering	The focal fish displaces the opponent with its mouth.
Submissive behavior	
Submissive display	
Submissive posture	The focal fish's body stands upright with its head up.
Submissive exhibition	The focal fish closes the tail and fins as a submissive posture.
Escape	
Escape from chase	The focal fish flees from the chase of the other fish.

Day 1: N = 12 males; four aquariums; three males per aquarium;
Day 2: N = eight males; four aquariums; two males per aquarium;
Two films per day (from 9:00 to 9:10 am and from 3:00 pm to 3:10 pm);
Focal fish: observed animal.

6.2.1.2 Experimental phase

Four males of each social status (dominant, subdominant, and submissive) were individually allocated to 12 aquaria in an CRD with three initial social statuses of males, one male with three females per aquarium (male to female ratio 1:3; FAO, 2009), and four replicates for each social status (Fig. 1). The thirty-six females selected had a mean initial weight of 7.52±1.55 g and a mean total length of 8.04±0.56 cm.

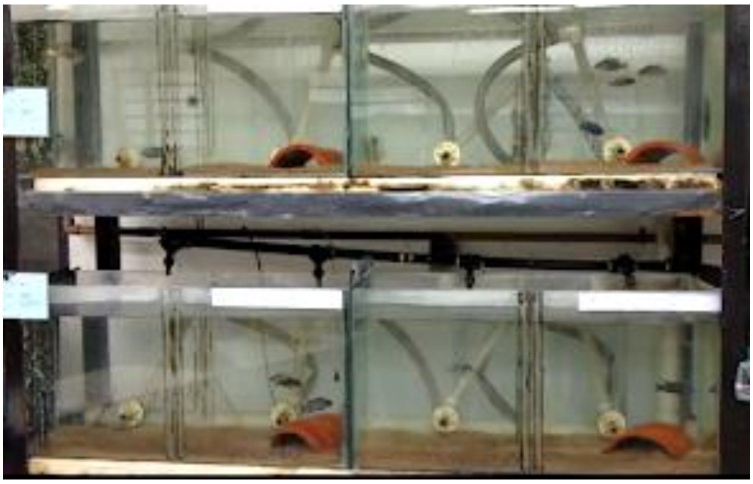

Fig. 1. Demonstration of the layout of the experimental units. In each experimental unit, one male of *Aulonocara nyassae* of each social status was allocated with three females. In the photo, there are four experimental units (aquariums), properly isolated by black adhesive between them, so there is no communication between the units.

The animals were recorded by a camera positioned in front of the tanks for 10 min at 09:00 and 15:00 h on days 6-10, 31-35 and 61-65 of the experiment in order to obtain data for two phases of the day and throughout the study period. Days 1–5 were reserved for adaptation of the animals to the aquariums, with behavioral observations not beginning until day 6. An ethogram was generated to record the reproductive behavior of males and females (Table 2).

All aquariums were enriched with a concave ceramic object (15 cm wide, 20 cm long, 12 cm high) and 3 cm of sand to cover the entire bottom of the to serve as shelter and to simulate the natural environment. The aim was to reduce more aggressive conflicts and facilitate nest building, courtship behavior, and spawning. Black polyethylene fabric was placed around the experimental environment, and black stickers were placed between aquariums to prevent movement and interaction around and between the aquariums from influencing the behavior of the animals.

Water quality parameters were measured three times a week. Dissolved oxygen was measured using a POL-69 Politerm oximeter (Politerm®, São Paulo, Brazil) and was maintained at 6.20±1.28 mg L^{-1}, while pH, temperature, salinity and conductivity were measured using a Hanna Instruments Combo PHEC\TDSHemperature HI98129 (Hanna Instruments Brasil®, São Paulo, São Paulo, Brazil) and were kept at 7.48±0.36, 27.80±0.63°C, 0.22±0.04 g L^{-1} and 0.44±0.08 mS cm^{-1}, respectively. Total ammonia and nitrite were measured using commercial colorimetric LabconTest® kits (Alcon®, Camboriú, Santa Catarina, Brazil) and were kept at 0.20±0.12 mg L^{-1} and 0.09±0.03 mg L^{-1}, respectively. Alkalinity was measured using the Amerimay Public Health Association (APHA) protocol (1998 Protocol) and was maintained at 112.68±8.90 mg L^{-1} of $CaCO_3$. The photoperiod was maintained at 12 h light and 12 h dark (12L:12D). Light intensity was measured weekly on the water surface of each aquarium using a digital luxmeter (Instrutemp® ITL 260; www.instrutemp.com.br) and was maintained at 260±50 lx.

Table 2. Ethogram for males of *A. nyassae* classified by different social status (dominant, subdominant and submissive) and their relationships with females in aquariums through filming every 30 days for 66 days.

Observation	Behavior description
Inactivity (male and female)	The fish remains in a stationary position.
Locomotion (males and females)	The fish moves from one point to another, without interaction with another fish.
Courtship	
Courtship (males and females)	
Lateral display	The focal fish exposes one side of the body towards the opposite sex.
Shivering	The focal fish flickers when it is close to the opposite sex.
Tail beat	Male and female stand side by side with the "S" shaped body.
Courtship (males)	
Chasing	The male chases the female.
Head-butting	The male presses the frontal region against the female.
Nest building	The male builds a nest in the sand to attract the female by removing the sand with his mouth below the shelter.
Going to nest	The male goes to the nest and moves his whole body to display.

Courtship (females)	
Female exit	The female escapes the male's approaches and swims to the other side of the aquarium.
Nest attraction	The female, attracted to the male, swims to the nest.
Attacking other females	The focal female attacks other female(s), opening and closing her mouth frequently.
Spawning	
Spawning (female)	
Sulcus construction	The female goes to the nest, built by the male, and creates small furrows in the nest.
Spawning	The female vibrates her caudal fins and releases the oocytes into the shelter.
Fertilization (male)	The male swims on top of the oocytes and releases semen
Parental care (females)	
Ventilation	The female moves the pectoral and pelvic fins close to the eggs.
Incubation	The female moves away from other animals present in the environment and protects the spawn in her mouth.

N=12 males; 12 aquariums; one male of each social status per aquarium;
N=36 females; 12 aquariums; three females per aquarium;
Every 30 days of the experiment, five days of observations (days 6, 7, 8, 9 and 10; 31, 32, 33; 34 and 35; 61, 62, 63, 64 and 65 of the experiment);
Two films per day (from 9:00 to 9:10 am and from 3:00 pm to 3:10 pm);
Focal fish: observed animal.

6.2.2 Growth and reproductive parameters

All animals (males and females) were measured and weighed at the end of 66 days using a Starrett 799 series digital caliper (150 mm capacity, ±0.02 mm accuracy; Starrett®) and Marte AS5500 digital balance (capacity 5 kg ±0.01 g accuracy; Marte Científica®), respectively.

Weight and length data were used to calculate:

- Initial weight (g) (IW);

- Final weight (g) (FW);

- Average weight gain (g) (AWG) = (FW - IW) ÷ number of animals per tank;

- Specific growth rate (% day^{-1}) (SGR) = SGR = 100 × [(ln FW - ln IW) ÷ Δt];

where IW is initial weight; FW is final weight and Δt is time between samplings.

- Initial mean total length (cm) (ITL);

- Final mean total length (cm) (FTL);

- Mean length gain (g) (MLG) = FTL- ITL;

- Fulton condition factor (K) = FW ÷ FTL^3.

Total survival was obtained after 66 days from direct counts transformed into percentages.

The mouths of females were observed daily for the presence of spawns. Eggs were collected five days after initial observation, which is one day before hatching. Eggs and larvae were maintained in a 27.4±1.2°C bath controlled by thermostat in containers containing 1 L of water at a density of up to 10 larvae L^{-1} and fed three times a day with a commercial mash diet containing 46% CP. Twice daily, before the first and last feeding, 50% of the water volume was siphoned and exchanged for clean water at the same temperature to maintain water quality. After 30 days, the larvae were anesthetized with Eugenol at 80 mg L^{-1}, counted, weighed, and measured (Ribeiro et al 2015). These data were used to evaluate:

- Average number of spawns per female, by social status of males = number of spawns ÷ number of females

- Average egg production per female, by social status of males = number of eggs ÷ number of females;

- Average egg hatching rate (%), per female, by social status = (number of total eggs - number of hatched eggs) ÷ number of females of each male social status;

- Mean number of larvae produced per female by social status = (number of larvae on day 1 - number of larvae on day 30) ÷ number of females of each male social status;

- Mean initial and final weight (g) of larvae by social status of males;

- Mean initial and final total length (mm) of larvae by social status of males.

6.2.3 Hemoglobin and blood biochemical variables

After 66 days, individual blood samples were collected from all surviving animals by caudal venipuncture. The fish were collected individually in the tanks and restrained with a damp cloth during blood collection. Heparin sodium (HEPAMAX-S® Cotia, São Paulo, Brazil) was used at 10% of the collected blood volume.

Hemoglobin concentration (g dL^{-1}) was determined by cyanometahemoglobin reaction using a Bioclin commercial kit (reference number K023-1) (https://bioclin.com QUIBASA® Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil).

The remaining aliquot of blood was centrifuged (Spinlab® SL-5 AM Centrifuge) at 1165 × g for 10 min for plasma separation and determination of glucose (mg dL^{-1}) by glucose oxidase test (GOD Trinder) (reference number K082-2); triglycerides (mg dL^{-1}) by Trinder monoreagent reaction (reference number K117); and cholesterol (mg dL^{-1}) by Trinder enzymatic method with a commercial kit (reference number K083-3). All tests mentioned above were analyzed by the Bioclin kit (https://bioclin.com QUIBASA® Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil). All analyses were read using a Biocrom Libra S22 spectrophotometer (Biocrom®; Cambridge, UK; https://analiticaweb.com.br). Plasma protein (g dL^{-1}) was measured using a RHC 200-ATC handheld refractometer (Huake Instrument Co.®; Zhejiang, China).

6.2.4 Somatic indexes

At 66 days, after blood collection, all animals were individually euthanized with eugenol at 285 mg L^{-1} (Mattioli et al., 2017) to obtain somatic indexes. The viscera of each animal were removed and weighed using a Marte AY220 digital balance (220 g capacity and ± 0.0001 g accuracy; Marte Científica®, São Paulo, São Paulo, Brazil). The liver, gonads and visceral fat were subsequently separated and weighed to obtain the following indexes:

- Visceral-Somatic Index (VSI): 100 × (viscera weight ÷ final weight);
- Hepatosomatic index (HSI): 100 × (liver weight ÷ final weight);
- Gonadosomatic index (GSI): 100 × (gonad weight ÷ final weight);
- Intraperitoneal fat index (IPFI): 100 × (visceral fat weight ÷ final weight).

6.2.5 Statistical analisys

All data (growth and reproductive performance, behavior, hemoglobin and serum biochemical parameters and organosomatic indexes) showed normality and homoscedasticity according to Shapiro-Wilk and Levene's tests, respectively, using Minitab statistical software (Minitab LLC® version 19; State College, Pennsylvania, USA). The data were submitted to ANOVA followed by Tukey's test ($P < 0.05$), and the results expressed in tables and figures as means ± standard deviation (SD).

6.3 Results

6.3.1 Ethogram

6.3.1.1 Social status of males

On day 1, only one male exhibited aggressive behavior in each of the four tanks, and thus was removed and classified as dominant ($P = 0.001$) (Fig. 2A). The other two males exhibited predominantly submissive behavior ($P = 0.03$) (Fig. 2B). On day 2 only one male exhibited predominantly aggressive behavior and was classified as subdominant ($P = 0.001$) (Fig. 2C), while the other male exhibited predominantly submissive behavior ($P = 0.04$) (Fig. 2D).

6.3.1.2 Reproductive behavior

The behaviors of inactivity ($P = 0.08$) (Fig. 3A) and fertilization ($P = 0.1$) (Fig. 3D) were not affected by social status throughout the experimental period. However, locomotion behavior was more frequent for subdominant and submissive males and less frequent for dominant males ($P = 0.003$) (Fig. 3B). Cutting behavior was most frequent in dominant and subdominant males and least for submissive males ($P = 0.02$) (Fig. 3C).

Female behaviors of inactivity ($P = 0.13$) (Fig. 3E), spawning ($P = 0.2$) (Fig. 3H), and parental care ($P = 0.07$) (Fig. 3I) were not influenced by male social status, however, locomotion (Fig. 3F) and courtship (Fig. 3G) were. Females with dominant and subdominant males performed locomotion more frequently ($P = 0.04$), while courtship was more frequent for females with dominant males, intermediate for females with subdominant males, and lower for females with submissive males (P = 0.04).

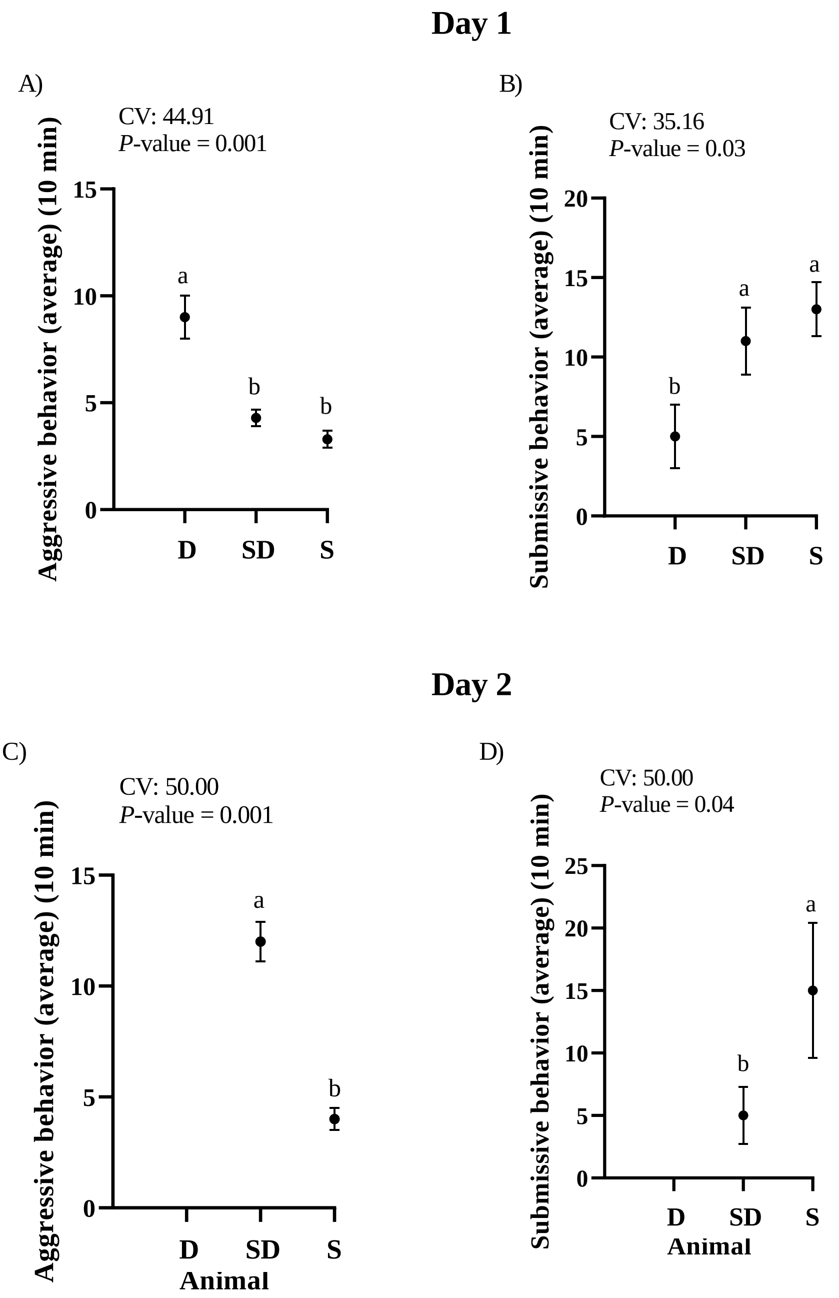

Fig. 2. Mean frequency of aggressive behavior (A) and submissive behavior (B) on day 1 (N = 3 males per aquarium), aggressive behavior (C) and submissive behavior (D) on day 2 (N = 2 males per aquarium) of males of A. nyassae filmed twice a day for 10 minutes.
Different letters in the columns indicate significant differences by the Tukey test (P < 0.05).
Data are expressed as mean values ± standard deviation of aggressive behavior
D: dominant; SD: subdominant; S: submissive.

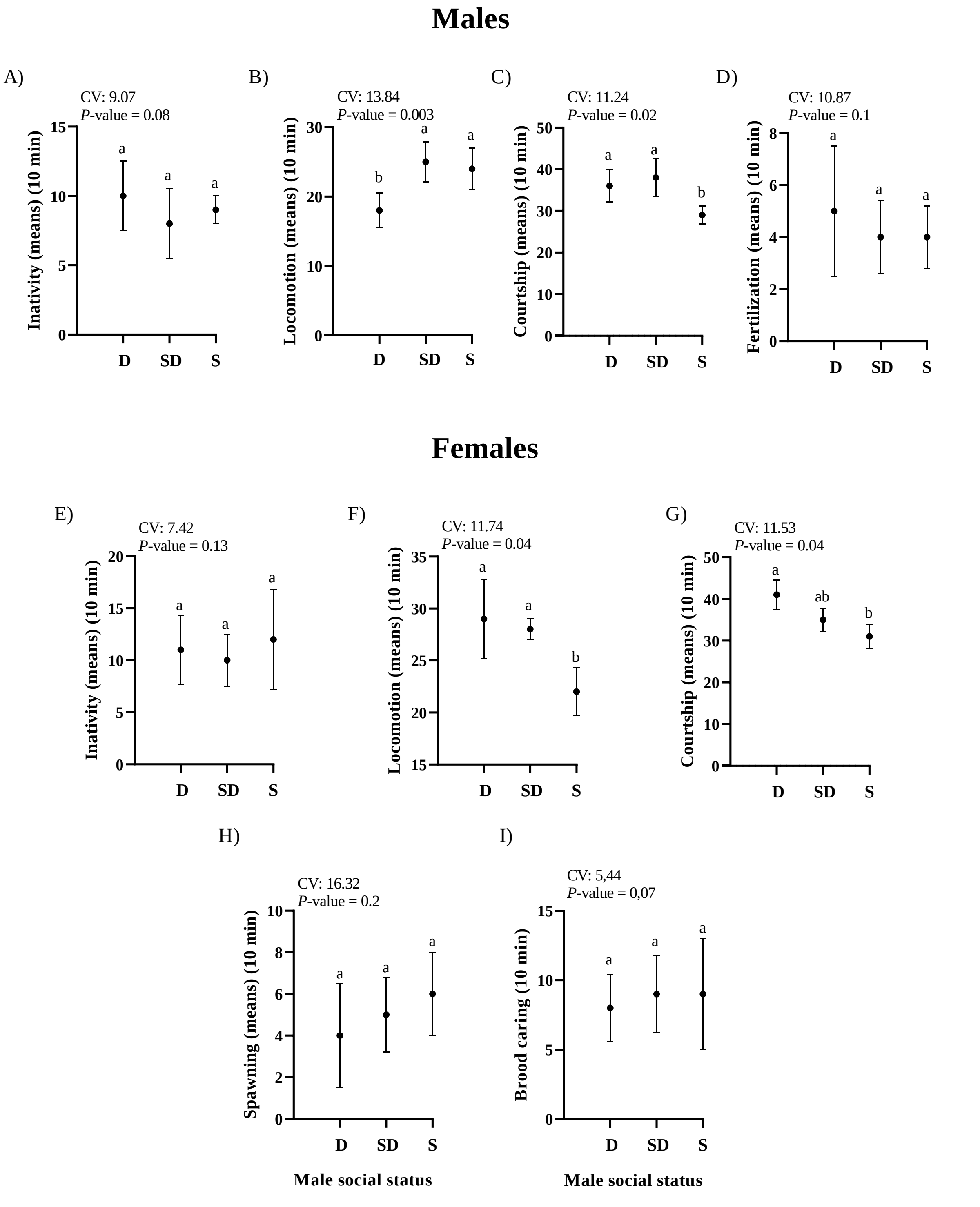

Fig. 3. Average frequency of inactivity (A), locomotion (B), courtship (C) and fertilization (D) of males and inactivity (E), locomotion (F), courtship (G), spawning (H) and parental care (I) of *A. nyassae* females filmed twice a day for 10 minutes with one male of each social status. Different letters in the columns indicate significant differences by the Tukey test ($P < 0.05$). Data are expressed as mean values ± standard deviation of aggressive behavior.
D: dominant; SD: subdominant; S: submissive.
N = 16 animals per treatment; four animals per aquarium; three females per aquarium; one male per aquarium.

6.3.2 Survival, growth and reproductive performance

There was no male mortality during the entire experimental period. The mean survival of females with dominant and submissive males was 100%, while female survival was lower with subdominant males (75.00 ± 14.43%) ($P = 0.007$).

For males, FW and AWG were higher for dominant animals and lower for subdominant and submissive animals ($P = 0.013$ and $P = 0.044$, respectively) (Table 3). However, SGR was highest for submissive males, followed by dominant and then subdominant ($P = 0.032$). There was no significant difference in FTL among treatments ($P = 0.161$), while ALG was higher for subdominant and submissive males and lower for dominant males ($P = 0.006$) and K was higher for dominant and submissive males and lower for subdominant males ($P = 0.035$).

For females FW, FTL and K were higher for animals kept with dominant and subdominant males and lower for those kept with submissive males ($P = 0.001$, $P = 0.0001$ and $P = 0.040$, respectively) (Table 3). However, AWG and FTL were higher for females with dominant males and lower for subdominant and submissive males ($P = 0.002$ and $P = 0.002$, respectively). SGR was highest for females kept with dominant males followed by those kept with submissive and then subdominant males ($P = 0.0001$).

For reproductive performance, mean spawning per female, mean initial weight, and mean initial larval length were not affected by male social status ($P = 0.432$, $P = 0.102$, and $P = 0.099$, respectively) (Table 4). On the other hand, mean egg production, hatching rate, and mean of number of larvae produced were higher for dominant and subdominant males and lower for submissive males ($P = 0.003$, $P = 0.022$, and $P = 0.018$, respectively). Mean final weight and mean final length were higher for larvae of dominant males and lower for subdominant and submissive males ($P = 0.05$ and $P = 0.002$, respectively).

Table 3. Final weight (FW) (g), average weight gain (AWG) (g), specific growth rate (SGR) (% day^{-1}), final mean total length (FTL) (cm), mean length gain (MLG) (cm) and Fulton condition factor (K) of males kept with three females per tank and females kept with males of different social status of *A. nyassae*, after 66 days of reproduction.

Male social status	Evaluated sex	FW (g)	AWG (g)	SGR (% dia $^{-1}$)	FTL (cm)	MLG (cm)	K
Dominant	**Male**	21.92±1.14[a]	10.53±1.30[a]	1.03±0.06[b]	10.65±0.25[a]	1.03±0.09[b]	1.97±0.21[a]
Subdominant		15.94±1.85[b]	6.83±1.55[b]	0.69±0.08[c]	9.80±0.30[a]	1.83±0.26[a]	1.52±0.10[b]
Submissive		17.34±1.96[b]	7.70±0.91[b]	1.32±0.11[a]	9.75±0.25[a]	2.13±0.26[a]	1.74±0.01[a]
CV (%)		16.49	24.91	26.71	4.89	30.85	14.01
***P*-value**		0.013	0.044	0.032	0.161	0.006	0.035
Dominant	**Female**	12.40±1.08[a]	4.75±0.75[a]	0.77±0.09[a]	9.38±0.38[a]	1.26±0.14[a]	1.58±0.04[a]
Subdominant		12.55±0.79[a]	2.98±1.04[b]	0.44±0.03[c]	9.26±0.29[a]	0.63±0.41[b]	1.60±0.09[a]
Submissive		9.21±0.79[b]	2.39±0.58[b]	0.60±0.03[b]	8.40±0.15[b]	0.46±0.10[b]	1.48±0.05[b]
CV (%)		17.44	37.84	25.01	5.89	55.22	5.47
***P*-value**		0.001	0.002	0.0001	0.0001	0.002	0.040

Different letters on the vertical indicate statistical differences by the Tukey test ($P < 0.05$);
Data are expressed as mean values ± standard deviation;
CV = coefficient of variation;
N males = four males per treatment;
N females = 12 females per treatment for dominant and submissive males; 9 females per treatment for subdominant males.

Table 4. Reproductive performance of females and zootechnical performance of progeny of *A. nyassae* from males of different social status, during 30 days of larviculture.

Male social status	Average number of spawns	Average egg production	Mean number of larvae produced (%)	Average of produced larvae (30 DAH)	Average initial weight of larvae (g)	Average final weight of larvae (g)	Average initial length of larvae (mm)	Average final length of larvae (mm)
	Female		***Larvae***					
Dominant	2.67±0.94[a]	31.75±2.15[a]	74.05±7.37[a]	24.00±4.24[a]	0.009±0.002[a]	1.06±0.064[a]	4.55±0.335[a]	29.25±1.89[a]
Subdominant	3.33±1.25[a]	29.57±2.94[a]	82.22±4.71[a]	23.17±4.37[a]	0.007±0.001[a]	0.83±0.096[b]	4.00±0.212[a]	24.5±1.29[b]
Submissive	2.00±1.00[a]	9.50±0.50[b]	53.74±11.66[b]	7.42±0.69[b]	0.006±0.001[a]	0.68±0.171[b]	3.85±0.497[a]	25.00±0.82[b]
CV (%)	46.15	47.73	22.19	56.00	26.07	21.97	11.49	0.82
***P*-value**	0.432	0.003	0.749	0.018	0.102	0.05	0.099	0.002

Different letters on the vertical indicate statistical differences by the Tukey test ($P < 0.05$).
Data are expressed as mean values ± standard deviation.
CV = Coefficient of variation
DAH: days after hatching

6.3.3 Hemoglobin and blood biochemical variables

Among males, total hemoglobin and plasma protein concentrations were higher for subdominant and submissive males relative to dominant males (P = 0.031 and P = 0.001, respectively) (Table 5), however, glucose was higher for dominant males and lower for subdominant and submissive males (P = 0.031). Triglyceride levels were higher for dominant and subdominant males and lower for submissive males (P = 0.041). Cholesterol was higher for submissive males and lower for dominant and subdominant males (P = 0.01).

Hemoglobin and plasma glucose concentrations were higher for females kept with dominant and lower for females kept with subdominant and submissive males (P = 0.0001 and P = 0.0001, respectively) (Table 5). However, the concentration of triglycerides and total plasma proteins was higher for females with submissive males and lower for females with dominant and subdominant males (P = 0.005 and P = 0.0001, respectively). Cholesterol concentration was higher for females with dominant and subdominant males and lower for females with submissive males (P = 0.0001).

6.3.4 Organosomatic indexes

For males, the VSI was highest for dominant males, followed by subdominant males and lowest for submissive males (P = 0.0001) (Table 5). The HSI was highest for dominant and subdominant males and lowest for submissive males (P = 0.021), while the GSI and the IPFI were higher for subdominant and submissive males and lower for dominant males (P = 0.014 and P = 0.017, respectively).

For females (Table 5), the HSI was higher for females with subdominant and submissive males and lower for females with dominant males (P = 0.027). The VSI, GSI, and IPFI were higher for females kept with submissive males and lower for those kept with dominant and subdominant males (P = 0.019, P = 0.001, and P = 0.0001, respectively).

Table 5. Hemoglobin (Hb) (g dL^{-1}), glucose (Glu) (mg dL^{-1}), triglycerides (TG) (mg dL^{-1}), total cholesterol (TC) (mg dL^{-1}), total plasma proteins (TPP) (g dL^{-1}), viscerosomatic index (VSI) (%), hepatosomatic index (HSI) (%), gonadosomatic index (GSI) (%) e intraperitoneal fat index (IPFI) (%) of males kept with three females per tank and females kept with males of different social status of *A. nyassae*, after 66 days of reproduction.

Male social status	Evaluated sex	Hb (g dL^{-1})	Glu (mg dL^{-1})	TG (mg dL^{-1})	TC (mg dL^{-1})	TPP (g dL^{-1})	VSI (%)	HSI (%)	GSI (%)	IPFI(%)
Dominant		6.19±0.90^{b}	52.56±3.53^{a}	147.58±14.38^{a}	85.13±14.38^{b}	4.18±0.19^{b}	11.78±0.38^{a}	2.43±0.21^{a}	0.25±0.01^{b}	0.82±0.02^{b}
Subdominant	**Male**	9.00±0.85^{a}	39.34±2.04^{b}	178.23±18.55^{a}	72.76±6.94^{b}	4.98±0.08^{a}	7.95±0.49^{b}	1.56±0.08^{b}	0.39±0.04^{a}	3.73±0.59^{a}
Submissive		8.25±0.71^{a}	43.57±1.25^{b}	105.65±1.34^{b}	135.22±31.47^{a}	5.25±0.15^{a}	6.06±0.20^{c}	2.92±0.16^{a}	0.42±0.02^{a}	4.33±0.40^{a}
CV (%)		22.65	13.64	22.28	34.62	6.37	25.13	25.41	20.67	53.71
***P*-value**		0.002	0.031	0.041	0.01	0.001	0.0001	0.021	0.014	0.017
Dominant		7.37±1.09^{a}	80.88±16.65^{a}	133.53±66.36^{b}	105.09±18.01^{a}	4.25±0.17^{b}	8.80±3.92^{b}	1.86±0.45^{b}	0.64±0.84^{b}	0.58±0.22^{b}
Subdominant	**Female**	5.01±0.93^{b}	42.74±7.50^{b}	108.13±48.24^{b}	128.32±7.84^{a}	4.28±0.17^{b}	10.73±3.34^{b}	2.79±0.49^{a}	0.60±0.24^{b}	0.53±0.08^{b}
Submissive		5.20±0.62^{b}	44.74±2.89^{b}	229.57±74.36^{a}	39.76±4.12^{b}	4.74±0.14^{a}	16.56±6.68^{a}	2.66±0.37^{a}	2.96±1.66^{a}	1.87±0.20^{a}
CV (%)		17.70	33.36	52.58	41.09	6.23	48.92	23.07	11.05	66.61
***P*-value**		0.0001	0.0001	0.005	0.0001	0.0001	0.019	0.027	0.001	0.0001

Different letters on the vertical indicate statistical differences by the Tukey test ($P < 0.05$).
Data are expressed as mean ± standard deviation.
CV = coefficient of variation.
N males = four males per treatment.
N females = 12 females per treatment for dominant and submissive males; 9 females per treatment for subdominant males.

6.4 Discussion

Behavior characteristic of the establishment of social status was observed in the present study and allowed males to be classified as dominant, subdominant or submissive.

Social status along a hierarchy determines physiological state and behavioral performance (Alward et al., 2020). A predominant feature of social hierarchies is the communication of classification through non-physical signaling systems (such as coloring) and aggression, characteristics that correlate with an individual's reproductive status (Alward et al., 2020).

Male *A. nyassae* showed aggressive behavior during the establishment of social statuses and reproduction, as also observed by Silva et al. (2021). The higher aggressiveness of dominant males was confirmed in the present study through the construction of an ethogram. This type of aggressive behavior was also reported for the Afrimay cichlids *O. niloticus* (Corrêa et al., 2003; Barreto et al., 2015) and *Astatotilapia burtoni* (Fulmer et al., 2017). Such aggressive relationships may be intensified by the conditions of captivity, wherein escape is limited to a small space (Barreto et al., 2015).

Males originally classified as subdominant were placed with females without the presence of other males. This new social conformation may have made the subdominant males more aggressive toward females. At the end of the experiment, males initially classified as subdominant and submissive experienced a change in coloration to the dominant blue. These results may be related to a rise in social status, even without competition between males. According to Parikh et al. (2006), when males ascend in status, there is an increase in aggression and body pigment and this change in coloration is important for male-female relationships. Males of the cichlid *A. burtoni* may be classified as dominant, intermediate (subdominant), and submissive (Fulmer et al., 2017). In addition, this process generates physiological changes in this same species. (Wallace et al., 2022). Subdominant males of this same species showed specific and different characteristics from dominant and submissive males, such as sudden changes in coloration and aggressiveness. These characteristics may have also influenced the results obtained for *A. nyassae*.

The social status of males influenced parameters of stress, growth, behavior and reproductive performance of females and males, as well as larviculture of the progeny. The stress that occurs during dominance settings between fish generates metabolic costs including blood and biochemical changes that negatively affect the immune system, growth and reproduction of the animals involved (Abbott et al., 2003). In addition, social interactions influence food intake and appetite of fish and lower food intake affects animal health (Eriegha and Ekokotu, 2017). The present work found a higher specific growth rate for submissive males and higher gonad length and weight gain for submissive and subdominant males. Since there was only one male per aquarium during the reproductive phase, dominance relationships may have been tempered. It has been reported that males show an increased growth rate with rising social status because there is less conflict and greater energy expenditure for growth and reproduction (Alonso et al., 2012; Silva et al., 2021).

Females with males classified as submissive showed reduced courtship behavior and moved less in the aquaria. The reduced locomotion of females with submissive males resulted in increased visceral fat deposition and liver and gonad weight. Although dominant and subdominant males moved less in the aquaria, they exhibited more courtship behavior, which may be associated with the protection of territory. Dominant males of *A. burtoni* were found to exhibit best timing and reproductive capacity (Renn et al., 2009). The same result was observed for *A. nyassae*, with dominant and subdominant males having better sperm quality and growth than submissive males (Silva et al., 2021). Although not observed for *A. nyassae* in the present study, when there was only one male *A. burtoni* per aquaria, submissive males have been reported to associate with and reproduce the behavior of females in the tanks (Hofmann et al., 1999).

Male social status influenced the growth performance and condition factor of female *A. nyassae* and the quality of progeny. The higher growth rate for females with dominant males had a positive influence on egg and larval quality, indicating that parents influence egg development, performance and larval survival, as reported by Izquierdo et al. (2001). It is possible that females mated with submissive males because they were the only alternative for reproduction, as there was only one male in the tank. In an environment with more males, however, dominant males would be the reproductive preference of these females. Thus, dominant males are better prepared for reproduction.

Silva et al. (2021) found that, at the ratio of one male to three females, the dominance status of male *A. nyassae* did not influence female performance or larviculture of progeny. However, the rearing tanks in that study were white and with no sand substrate while those of the present study were glass with black sides and sand substrate. Tank color influences reproduction and husbandry performance of fish, and white color may make fish more aggressive (McLean, 2020). In an experiment on dominance status and environment color, Luchiari et al. (2007) reported that juvenile and adult *O. niloticus* prefer yellow environments. Thus, sandy substrates and black sides may be more favorable environments for *A. nyassae* reproduction.

Regarding hematological and biochemical parameters, dominance relationships are an important stress-related factor for *O. niloticus* and are known to influence metabolic parameters in dominant and subordinate fish (Corrêa et al., 2003). All parameters evaluated for males and females in the present study were influenced by the social status of *A. nyassae* males. Plasma proteins, in addition to being indicators of stress in fish, are also related to hemoglobin transport (Saunders, 2013). Hemoglobin levels rise in stress situations to improve oxygen transport capacity (Misra et al., 2006). The present study found higher concentrations of hemoglobin and plasma protein for subdominant and submissive males. For females, however, it was the reverse, with a higher concentration of hemoglobin for those with dominant males and plasma protein for those with submissive males. Therefore, subdominant and submissive males and females with dominant males required higher hemoglobin concentrations and females with submissive males required higher plasma protein concentrations because they moved more than dominant males during the experimental period and may have generated greater stress.

Cholesterol is a precursor of steroid hormones, and its serum levels affect gonadal development and oocyte production (Lubzens et al., 2010). Despite the higher cholesterol concentration in submissive males and in females with dominant and subdominant males, the highest reproductive capacity was detected for dominant males. The lower cholesterol level for dominant males may be related to aggressiveness. It is reported that low cholesterol concentrations are associated with aggressive behavior because there is decreased serotonin activity and dominant animals possibly used more cholesterol to synthesize more cortisol, which also has an effect on aggressiveness (Kaplan et al., 1997). For females, the higher cholesterol concentration with dominant males may be related to the greater reproductive stimulus that dominant males may exert on them. This influence exerted by males on females is related to the chemical, visual, and acoustic communication that occurs between animals at the time of courtship (Huertas et al., 2008; Huertas et al., 2014).

Glucose levels were higher for dominant males and for females with dominant males. Variation in glucose may promote changes in energy balance and, therefore, may influence physiological and reproductive processes (Shahjahan et al., 2014). Dominant males had greater cutting behavior, a movement that may have generated more stress, which in turn justifies the higher concentration of glucose in the blood of these animals.

Triglycerides were higher for dominant and subdominant males and for females with submissive males. Male *O. niloticus* in the dominance establishment phase experienced no changes in glucose, triglycerides and total protein levels among the different social statuses, thus, dominant and submissive individuals have the same intensity of stress and metabolic damage (Corrêa et al., 2003).

When these males are isolated, glucose, triglycerides and total protein levels are lower than during the dominance establishment phase (Corrêa et al., 2003). The higher concentration of glucose for dominant males may have acted to save fat and probably increased body lipid reserves as demonstrated by higher triglycerides levels, greater weight gain, higher liver weight and lower locomotion behavior. In addition, the higher triglycerides level in females with submissive males may be associated with higher fat deposition in the viscera, lower husbandry performance and lower offspring quality. As observed, female growth, egg production and hatching rate, and the number of larvae produced was lower for submissive males. Therefore, the different social conformations related to dominance status affect blood parameters and, thus, it is not advisable to use submissive males as broodstock for *A. nyassae*.

6.5 Conclusions

Male *A. nyassae* may be considered aggressive during the establishment of social status and during reproduction. Moreover, even after division and separation by social status, males still exhibit status-specific behaviors.

The social status of males influences the performance, stress responses, energy metabolism, and reproductive capacity of males and females of *A. nyassae*. As a consequence, progeny quality is also influenced by the social status of males. Therefore, the use of males classified as dominant as breeders is recommended since they induce higher female growth through the social communication that exists between animals and improved progeny quality through genetic influences.

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7. CONSIDERAÇÕES FINAIS

O ciclídeo africano *A. nyassae* apresenta coloração azul e há interesse como uma espécie de peixe ornamental. Como a maioria dos machos de ciclídeos, o *A. nyassae* apresenta comportamento agressivo durante o estabelecimento das relações de dominância. Esse *status* de dominância é influenciado pela quantidade de machos e fêmeas presentes no mesmo ambiente. Os animais dominantes são mais agressivos e apresentam coloração azul mais acentuada, na qual, o mercado tem mais interesse. Além disso, machos dominantes e subdominantes apresentam melhor qualidade espermática e esses *status* podem influenciar o comportamento, fisiologia e desempenho zootécnico e sucesso reprodutivo de fêmeas e a qualidade das larvas produzidas de forma positiva.

O presente estudo contribuiu para novos resultados relacionados ao manejo produtivo de *A. nyassae* em cativeiro, como, separação dos animais de acordo com *status* social, coleta de ovos e larvas. Portanto, traz novas informações aos produtores e vendedores desta espécie e novos conhecimentos para o aprimoramento da aquicultura de ciclídeos africanos ornamentais. Além disso, as análises de qualidade espermática, parâmetros hematológicos e perfil bioquímico são inéditas para espécies do gênero *Aulonocara*, importante para a aquicultura ornamental e é base para estudos associados à reprodução e *status* social para as diferentes espécies de ciclídeos.

Outros estudos sobre o comportamento social e reprodutivo de *A. nyassae*, relacionados à *status* sociais em diferentes densidades de estocagem, temperaturas, cores de tanque, tipos de substratos e ninhos, intensidades luminosas e fotoperíodos podem ser executados para aprimorar ainda mais os protocolos de produção e bem-estar desses animais.

ANEXOS

ANEXO A – Artigo I (primeira página)

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Influence of social status on growth performance, reproductive success and sperm quality of the African cichlid *Aulonocara nyassae*

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A R T I C L E I N F O

Keywords:
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Submissive
Reproduction
Sperm quality

A B S T R A C T

The status of social dominance may have consequences for the well-being of cichlids. These fish show territorial and aggressive behavior and are important for behavioral studies. The aim of the present study was to evaluate sperm variables of motility, growth performance and reproductive performance according to the social status of African cichlid *Aulonocara nyassae*. The experiment was divided into: Phase 1 - establishment of social statuses between males, 40 males of *A. nyassae* were kept in four tanks (42 L useful volume) with 10 animals in each tank for 30 days. During this period, the animals were classified according to social status as dominant, subdominant and submissive fish; Phase 2 – reproduction, one male of each social status (dominant, subdominant and submissive) per tank was selected, for a total of four males for each status and a total of 12 males. One male of each social status of each of original four tanks (total 12 males) was placed in a new tank with three females, for a total of 12 new tanks, and; Phase 3 - individualization of males (removal of females), the males were separated from their females and placed individually in 12 tanks. In Phase 1, only one dominant male was detected in a group of 10 animals, between 6 and 7 subdominant animals and, 2 and 3 submissive animals. The final weight (FW) was higher for dominant fish. The sperm concentration, motility rate (MOT), Amplitude of lateral head movement (ALH), linearity (LIN), straightness (STR), wobble (WOB) and beat-cross frequency (BCF) did not show statistical difference between dominant and subdominant fish. Curvilinear velocity (VCL), Straight-line velocity (VSL) and Average path velocity (VAP) were higher for subdominant fish. Motility time was higher for the dominant male. In phase 2, the male performance was higher for dominant and subdominant than submissive males. MOT and VAP were higher for dominant males. ALH, LIN, STR, WOB and BCF were higher for subdominant males. The social status of the males did not influence the growth and reproductive variables of the females. In phase 3, the male performance was higher for dominant and subdominant fish. MOT, VCL, VSL, VAP and WOB were superior to dominant and subdominant males. LIN and STR were superior for subdominant males. BCF was superior to dominant. Therefore, sperm quality and growth performance were influenced by changes in the social statuses.

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ANEXO B – Artigo II (primeira página)

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Influence of the social status of male *Aulonocara nyassae* on behavior, growth, hematology, biochemical parameters, reproduction and larviculture

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Lake Malawi
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ABSTRACT

The aim of the present study was to evaluate the influence of social status of *Aulonocara nyassae* males on behavior, growth and reproductive performance, hematological parameters and male and female biochemicals and the success of offspring larviculture. For the pre-experimental phase, four aquariums with three males each were observed for 48 h and videos of 10 min, twice a day, were recorded and an ethogram was generated on the definition of social status, classified according to aggressive behavior, territorialism and coloration as dominant, subdominant and submissive. The experimental phase lasted 66 days. One male was stocked per aquarium, from each of the three social statuses, with three females and four replicates. The animals were filmed for 10 min, twice a day, for 5 days, every 30 days. An ethogram on the reproductive behavior of males and females was generated. Locomotion was more frequent in subdominant and submissive males and in females with dominant and subdominant males ($P < 0.05$). Courtship behavior was more frequent in dominant and subdominant males and in females from aquariums with dominant males ($P < 0.05$). The survival of females with subdominant males was lower ($P < 0.05$). Final weight (FW), average weight gain (AWG), glucose (GLU), triglycerides (TG), viscerosomatic (VSI) and hepatosomatic (HSI) indices for males; final mean total length (FTL), mean length gain (MLG), hemoglobin (Hb), specific growth rate (SGR), Fulton condition factor (K), total cholesterol (TC), FW, AWG, GLU and for females and for progeny, average of egg production, hatching rate, average of larvae produced, mean final larvae weight and mean final larvae length were higher for dominants. In males, SGR and FTL, in females, total plasma protein (TPP), gonadosomatic index (GSI), intraperitoneal fat index (IPFI), TG, HSI and VSI were higher for submissive ($P < 0.05$). However, the mean length gain (MLG), GSI and IPFI, Hb and TPP were higher for subdominant and submissive males ($P < 0.05$). K was higher for dominant and submissive males ($P < 0.05$). Therefore, the social status of males influences growth performance, hematological, biochemical parameters and reproductive capacity of males and females of *A. nyassae*.

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