UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CENTRO DE AQUICULTURA DA UNESP

ARGININA NA DIETA DE REPRODUTORES DE *Rhamdia quelen*

Danielle Zanerato Damasceno

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Doutoranda: Danielle Zanerato Damasceno

Orientadora: Dra Elizabeth Romagosa

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: ARGININA NA DIETA DE REPRODUTORES DE *Rhamdia quelen*

AUTORA: DANIELLE ZANERATO DAMASCENO
ORIENTADORA: ELIZABETH ROMAGOSA
COORIENTADOR: FÁBIO BITTENCOURT

Aprovada como parte das exigências para obtenção do Título de Doutora em AQUICULTURA, pela Comissão Examinadora:

Profa. Dra. ELIZABETH ROMAGOSA
Centro de Pesquisa em Peixes Ornamentais / Instituto de Pesca, IP, São Paulo-SP

Prof. Dr. SERGIO RICARDO BATLOUNI
Laboratório de Reprodução de Peixes / Centro de Aquicultura da UNESP, CAUNESP

Pesquisador GIOVANI SAMPAIO GONCALVES
Centro do Pescado Continental / INSTITUTO DE PESCA

Prof. Dr. EDUARDO ANTONIO SANCHES
Engenharia de Pesca / Câmpus Experimental de Registro/ UNESP (por meio de Videoconferência)

Profa. Dra. TAÍS DA SILVA LOPES
Departamento de Aquicultura / Instituto Pesca, APTA, São Paulo-SP

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Sumário

O fardo é proporcional às forças, assim como a recompensa será proporcional à resignação e à coragem.

Allan Kardec

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Lista de abreviaturas

ARG – arginina, *arginine*

NO – óxido nítrico, *nitric oxide*

NOS – óxido nítrico sintase, *nitric oxide synthase*

iNOS – óxido nítrico sintetase induzível, *inducible nitric oxide synthase*

eNOS – óxido nítrico sintetase endotelial, *endothelial nitric oxide synthase*

nNOS – óxido nítrico sintetase neural, *neural nitric oxide synthase*

NADPH - Nicotinamida Adenina Nucleótido fosfato, *Nicotinamide adenine dinucleotide phosphate*

GSI – índice gonadosomático, *index gonadosomatic*

HSI - índice hepatossomático, *index hepatosomatic*

VGI – índice de gordura visceral, *visceral fat index*

CASA – *Computer Assisted Sperm Analysis*

A_{und} – cistos de espermatogônia indiferenciada tipo A, *cysts of type A undifferentiated spermatogonia,*

A_{diff} – cistos de espermatogônia diferenciada tipo A, *cysts of type A differentiated spermatogonia,*

SPG B – cistos de espermatogônia tipo B, *cysts of type B spermatogonia,*

SPC – cistos de espermatócitos, *cysts of spermatocytes,*

SPT – cistos de espermatide, *cysts of spermatid*

cAMP – adenosina monofosfato cíclica, *cyclic adenosine monophosphate*

RESUMO

Informações sobre a nutrição de reprodutores são fundamentais para que haja uma boa performance reprodutiva, e maior qualidade na produção de gametas e prole, entretanto esse tipo de estudo ainda não é realiado em grande quantidade. Com base nisso este trabalho teve como objetivo investigar o efeito da arginina na nutrição de reprodutores de *Rhamdia quelen*. Para isso 800 juvenis da espécie foram alimentados durante cinco meses com dietas que continham diferentes níveis de arginina (1,37; 1,67; 1,97; 2,27 e 2,57%). Após este período reprodutores de cada tratamento foram selecionados e induzidos hormonalmente (2.5 $mg.kg^{-1}$ Extrato Hipofisário de Carpa para machos e 5.5 $mg.kg^{-1}$ para fêmea), após 240 horas-grau foi realizada a coleta de sêmen e ovócitos. No sêmen foi avaliado: volume, pH, concentração, motilidade e velocidade espermática, normalidade, e foi aferido o diâmetro do ovócitos. As gônadas, fígado e gordura visceral de ambos os sexos foram removidas e pesadas para cálculo dos índices somáticos, e foi realizada a fertilização, incubação e após a abertura da boca as larvas foram transferidas para caixas de plástico onde foi realizada a larvicultura durante 10 dias. Foi realizada quantificação de: vitelogenina no plasma sanguíneo de fêmeas e de óxido nítrico nas gônadas de machos e fêmeas. Verificamos que a suplementação com 2,27% de arginina influenciou a produção seminal, tanto em volume quanto em concentração, ao mesmo tempo que promoveu a produção de ovócitos com maior diâmetro, que consequentemente gerou larvas mais resistentes. Além disso houve maior produção de óxido nítrico nas gônadas. Concluímos com estes resultados, apesar de iniciais, que a arginina tem um importante papel nos gametas e que mais estudos devem ser executados para uma melhor compreensão da função e ação na fisiologia reprodutiva.

PALAVRAS-CHAVE: aminoácido, nutrição de reprodutores, larvicultura, ovócitos, sêmen.

ABSTRACT

Information about broodstock nutrition are essential for a good reproductive performance, and higher quality in gamete and offspring production, however this type of study is still not performed in large quantity. With the objective of assessing the effect of arginine on the reproduction of *Rhamdia quelen* females, 800 fish were fed for five months with diets containing 1.37, 1.67, 1.97, 2.27 and 2.57% arginine for seven months. After this period the fish of each treatment were selected and received hormonal induction (2.5 $mg.kg^{-1}$ carp pituitary extract for males and 5.5 $mg.kg^{-1}$ for females), after 240 termal units accumulated the semen and oocytes were collected. The semen were collected and analysed: volum, pH, concentration, velocity, motility and normality, and the diameter of oocytes. The gonad, liver, visceral fat of both sexes were collected to somatic analysis. The fertilization, incubation and after the larvae open the mouth were transfered to plastic box were the larviculture was carried for 10 days. Were analysed the production of vitellogenin in bood plasm of female and nitric oxide on gonad of males and females. We observed that the addition of 2.27% arginine influenced the seminal production, both in volum and concentration, at the same time that it promoted the oocyte production ith bigger diameter, that consequently generated more resistant larvae. The addition of 2.27% arginine to the diet of *R. quelen* females favored reproductive parameters, which suggests that arginine increases the nitric oxide production, and consequently raises reproductive efficiency. In addition also promoted the higher nitri oide production. We concluded that these results, although initials, that the arginine present an important role in the gametes and that more studies should be performed for a better understanding of the function and action in the reproductive physiology

KEY-WORDS: amino acids, broodstock nutrition, larviculture, oocyte, semen.

1. Introdução geral

O *Rhamdia quelen*, popularmente conhecido como jundiá, é uma espécie de alto interesse comercial, principalmente na região Sul do Brasil, devido a características como o rápido crescimento, fácil adaptação a ambientes e dietas distintas, além de apresentar facilidade no manejo (Barcellos et al., 2004; Parra et al., 2008). Outra característica valorizada é que a espécie não apresenta redução no ganho em peso durante o inverno, o que possivelmente indica a resistência do *R. quelen* a baixas temperaturas (Fracalossi et al., 2004), além de apresentar fácil resposta quando submetidos a reprodução artificial.

A nutrição de reprodutores é continuamente estudada em mamíferos e espécies com potencial zootécnico como suínos (Brooks & Cole, 1974, Prunier & Quesnel 2000, Wu et al., 2012, Ren et al., 2017), bovinos (Sartori et al., 2016, Birne et al., 2017) e aves (Surai & Fisinin, 2014). Entretanto, estudos que avaliam a nutrição de reprodutores de peixes ainda são escassos.

A reprodução de peixes pode ser controlada por inúmeros fatores, como hormonais, ambientais, sociais e nutricionais (Zhang et al., 2013), e no caso de *R. quelen* informações descritas por Parra et al. (2008), Reidel et al. (2010), Coldebella et al. (2011), Tessaro et al. (2012 a e b), Diemer et al. (2014), mostram que a nutrição realmente reflete no desenvolvimento gonadal, a qualidade dos gametas e da prole quando recebem dietas experimentais com níveis ideais de proteína bruta, energia digestível, lipídeos distintos e lisina, que até o momento foi o único aminoácido testado.

A exigência de aminoácidos essenciais para peixes vem sendo investigada há mais de 50 anos, entretanto, muitas vezes é superestimada para aminoácidos que são preferencialmente depositados na proteína corporal como leucina e lisina, enquanto a exigência de aminoácidos que tem papel fundamental no metabolismo, como, metionina, treonina, histidina e arginina são subestimados (NRC, 2011).

Pesquisas sobre o papel dos aminoácidos nos processos fisiológicos relacionados a nutrição de peixes são realizados com frequência e mostram a importância destes componentes na dieta. Entre os aminoácidos estudados encontra-se a arginina que tem seu efeito avaliado principalmente para o desempenho e imunidade de espécies com potencial zootécnico na aquicultura como: tilápia do Nilo, *Oreochromis niloticus* (Neu et al., 2016, Pereira et al., 2017), catfish amarelo, *Pelteobagrus fulvidraco* (Chen et al.,

2016), linguado, *Scophthalmus maximus* (Zhang et al, 2017), bagre do canal, *Ichtalurus punctatus* (Pohlenz et al., 2013), striped bass hibrido (Cheng et al., 2012). Entretanto até o momento seu efeito na reprodução de peixes é desconhecido.

A arginina é um aminoácido funcional (Wu, 2009) que atua nas vias metabólicas necessárias para a manutenção, crescimento, reprodução e imunidade dos organismos (Suenaga et al., 2008). A arginina além de constituinte de proteínas, ainda está envolvida na síntese de poliaminas e prolina (Nikolic et al., 2007), como substrato para a síntese do colágeno e de óxido nítrico, considerada como um componente chave do plasma seminal e espermatozoides (Lahnsteiner, 2009). E é um aminoácido essencial para jovens animais que experimentam um rápido crescimento (Wu et al., 2009). Para peixes de água doce, o ciclo da ureia – um caminho para a síntese da arginina – é muito insipiente comparado com mamíferos, de modo que a deficiência de arginina afeta o crescimento e a retenção de proteína em peixes (Abidi & Khan, 2009).

A arginina é um dos aminoácidos que sintetizam as poliaminas, dentre elas as poliaminas espermina e espermidina apresentam papel fundamental na espermatogênese (Wu et al., 2008). Sabe-se que as poliaminas atuam nas funções reprodutivas dos mamíferos, e atuam na regulação da esteroidogênese ovariana durante o ciclo estral e gravidez, tornando-se indispensáveis na embriogênese (Lefèvre et al., 2011). Além disso, o incremento de arginina na dieta de mamíferos indica os benefícios deste aminoácido também para a melhoria de características reprodutivas masculinas. Estudos realizados com homens inférteis mostram que a arginina na alimentação promove o aumento a motilidade dos espermatozoides (Scibona et al., 1994). Outro estudo com humanos mostra que a incorporação de micronutrientes, inclusive arginina, promove o aumento do volume seminal, da concentração e da normalidade espermática (Imhot et al., 2012). A arginina desempenha papel estimulante na motilidade espermática de humanos, coelhos e cabras (Aydin et al., 1995, Radany et al., 1981, Patel et al., 1998, Srivastava et al., 2006), e sua deficiência na dieta pode causar perda da motilidade espermática em homens, além de diminuir expressivamente a quantidade de espermatozoides (Wu et al., 2008).

Os estudos que avaliam a suplementação de arginina na dieta de reprodutores atribuem o sucesso dos resultados ao óxido nitrico. Este é, fisiologicamente, um importante gás sinalizador, versátil e onipresente sintetizado pela arginina (Rosseli et al 1998), e apenas recentemente passou a ser investigado na reprodução de peixes e apesar

do número limitado de estudos apresenta-se como componente importante na regulação da espermatogênese e produção hormonal (Lal e Dubey, 2013, Singh e Lal 2017).

Os resultados encontrados em publicações científicas sobre o efeito positivo na reprodução após a inclusão de arginina na dieta de mamíferos aliada a escassez de estudos sobre a nutrição de reprodutores de peixes e a inexistência de informações sobre o efeito da arginina na reprodução de peixes foram os principais motivos para a realização deste estudo.

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2. REVISÃO BIBLIOGRÁFICA

2.1.Nutrição de reprodutores de peixe

A nutrição de reprodutores é continuamente estudada em mamíferos e espécies com potencial zootécnico como suínos (Brooks & Cole, 1974, Prunier & Quesnel 2000, Wu et al., 2012, Ren et al., 2017), bovinos (Sartori et al., 2016, Birne et al., 2017) e aves (Surai & Fisinin, 2014). Entretanto, estudos que avaliam a nutrição de reprodutores de peixes ainda são pouco realizados. Este tema ainda levanta inúmeras questões, embora os estudos publicados indiquem a relação direta entre nutriente e qualidade na reprodução. Sendo que a limitação da produção de dietas para peixes na fase reprodutiva ainda é fator que prejudica o desenvolvimento da aquicultura (Morais et al., 2014).

Pesquisas com nutrição de reprodutores de espécies marinhas e de água doce vem sendo realizadas e mostram bons resultados para tanto machos quanto para fêmeas. De acordo com Izquierdo et al. (2001) a embriogênese e o desenvolvimento inicial das larvas, antes mesmo da absorção completa do saco vitelínico, é fortemente dependente da nutrição dos reprodutores. Durante o desenvolvimento ovariano as reservas maternas e nutrientes da dieta são mobilizados para os oócitos e servirão para suprir nutricionalmente o embrião até o inicio da alimentação exógena (Fontagné-Dicharry et al., 2017). Izquierdo et al. (2001) reporta que a fecundidade reduzida de algumas espécies pode ser associada ao desbalanço nutricional, que pode causar desequilibrio no eixo cérebro-pituitaria-gônadas ou restrição de componentes bioquimicos na formação dos ovos.

Em estudos com nutrição de machos reprodutores é observado que a qualidade seminal também é relacionada com a dieta. Pesquisas sobre a suplementação de

aminoácidos como lisina, metionina, vitaminas, diferentes tipos de óleos mostram o efeito dos compostos na produção de gametas e no desenvolvimento inicial de larvas (Diemer et al., 2014, Fontagné-Dicharry et al., 2010, 2017, Morais et al., 2014).

Durante o processo de vitelogênese, nutrientes como aminoácidos são transferidos do fígado para os oócitos por meio do fluxo sanguíneo (Fontagné-Dicharry et al. 2017). De acordo com Tandler et al. (1995) a nutrição de reprodutores com adequado balanço de aminoácidos promove uma melhor síntese de vitelogenina, e por sua vez o sucesso do desenvolvimento incial das larvas de peixes está relacionado ao balanço de aminoácidos presentes nos ovos (Brooks et al. 1997; Srivastava et al., 1995). Seguindo nessa mesma linha de pesquisa, Fontagné-Dicharry et al. (2017) verificaram que a metionina na nutrição de trutas arco-íris influencia na qualidade da prole, enquanto Seilez et al. (2017) constataram que sua deficiência influencia negativamente. Bittencourt et al. (2018) notaram que a suplementação adequada de lisina na dieta de fêmeas de *Rhamdia quelen* promove benefícios nos parâmetros reprodutivos e nos valores de fecundidade absoluta, enquanto Reidel et al. (2010) mostraram a adequada relação entre proteina bruta/energia digestivel (35%/3250 kcal.g^{-1}) influencia na maturação final das gônadas e no processo de vitelogênese. Masoudi Asil et al. (2017) verificaram que a suplementação de 2% de ácido aracdônico em *Trichopodus trichopterus*, que é um peixe modelo para experimentos, aumentou os valores de fecundidade, o diâmetro do saco vitelínico e a taxa de eclosão. Para fêmeas de tilápia do Nilo, Bombardelli et al. (2017) observaram que dietas contendo 280 $g.kg^{-1}$ proteína digestível e 16,74 MJ kg^{-1} energia digestível promoveram a produção de um número baixo de ovos, entretanto, este valor é compensando pelo maior diâmetro dos ovos que promovem larvas com maiores chances de sobrevivência.

A nutrição reprodutores do sexo masculino também é influencia nos resultados da reprodução. Segundo Cabrita et al. (2014) dietas de qualidade promovem a produção de sêmen e espermatozoides de melhor qualidade. Entre os aminoacidos estudados na nutrição de machos reprodutores encontra-se o triptofano, que segundo Akiyama et al., 1996 a suplementação de 0.1% na dieta de *Plecoglossus altielis* melhora a produção de testosterona e prolonga o tempo de espermiação. Diemer et al. (2014) verificaram que a lisina na alimentação de *Rhamdia voulezi* promove aumento no volume seminal. Xu et al.

(2017) verificaram que a suplementação com acido aracdônico aumenta a produção de testosterona em machos em *Cynoglossus semilaevis*.

Embora saiba-se pouco sobre o efeito a longo prazo da nutrição de reprodutores sobre a prole durantes fases avançadas de desenvolvimento (juvenis e adultos) (Izquierdo et al., 2015), os resultados acima citados mostram a importância da investigação da nutrição de reprodutores, e deixa claro o poder que uma alimentação balanceada representa sobre a qualidade dos gametas, masculinos e femininos e, consequentemente, sobre a prole. E também abre espaço para a necessidade de um número cada vez maior de estudos, garantindo informações precisas e que garantam o sucesso reprodutivo para incontáveis espécies de peixes.

2.2.Aminoácidos

Os aminoácidos exercem inúmeras funções, e agem na manutenção do metabolismo, crescimento, reprodução e respostas imunes (Meijer, 2003; Li et al. 2009). E são definidos como subtâncias orgânicas que contêm o grupo ácido e o amino (Brody, 1999). A exigência de aminoácidos essenciais para peixes vem sendo investigada há mais de 50 anos, entretanto, muitas vezes é superestimada para aminoácidos que são preferencialmente depositados na proteína corporal como leucina e lisina, enquanto a exigência de aminoácidos que tem papel fundamental no metabolismo, como, metionina, treonina, histidina e arginina são subestimados (NRC, 2011).

Proteina é o componente mais caro da ração utilizada na produção animal, incluindo para animais aquáticos (Wilson et al., 2002), além de ser indispensável para todos os organismos vivos, incluindo os peixes (Monentcham et al., 2010). Em geral, uma proteína contém 300 aminoácidos, apresenta inúmeras funções estruturais e metabólicas e é essencial à qualquer tipo de célula no corpo (NRC, 2011). Os peixes não apresentam uma exigência específica de proteínas, mas sim uma suplementação equilibrada de aminoácidos essenciais e não-essenciais (Monentcham et al., 2010). Os valores de proteína bruta são importantes, mas não garantem que haja a qualidade adequada de proteína, portanto se faz necessária a quantificação e suplementação ideal de aminoácidos essenciais e não-essenciais (Monentcham et al., 2010).

Com o passar dos anos e com o avanço da pesquisa em peixes anseia-se a formulação de dietas cada vez mais baratas, e que causem o mínimo impacto sobre a

qualidade da água e utilize como fonte proteica alimentos de origem vegetal. Para isso, inúmeros trabalhos tem sido realizados para definir a exigência de aminoácidos para a diferentes espécies de peixes, incluindo a avaliação de ovos e larvas que dependem do equilibrio aminoacídico como energia para o metabolismo (Fyhn, 1993). A determinação das exigências de aminoácidos pode ser realizada por dois métodos, sendo um dose-resposta (Diemer et al., 2014, Pereira et al.,2017, Zaminhan et al., 2017, Bittencourt et al., 2018) e o outro, pela avaliação da composição de aminoácidos do corpo inteiro ou ovos (Meyer & Fracalossi 2005, Monentcham et al., 2010).

Baseados no crescimento e balanço de nitrogênio, os aminoácidos são classificados como essenciais e não-essenciais, tanto para animais quanto para humanos (Li et al., 2009, Wu, 2009). Os aminoácidos essenciais são os que não são sintetizados de forma eficiente ou em quantidade suficiente para atender as necessidades do animal, e que devem ser suplementadas na dieta. Enquanto isso, os aminoácidos não-essenciais são aqueles que são sintetizados pelo organismo em forma e quantidade suficientes (Wu, 2009), para peixes os aminoácidos essenciais são arginina, histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano e valina e os não essenciais são : alanina, asparagina, aspartato, cisteina, glutamato, glutamina, glicina, prolina, serina, taurina e tirosina. Além de atuarem na construção de proteínas e polipeptídeos, alguns aminoácidos tem o papel regulador nas rotas metabólicas necessárias para o crescimento, reprodução, imunidade e, assim por diante, são chamados de aminoácidos funcionais (arginina, cisteina, glutamina, leucina, prolina e triptofano) segundo Wu (2010). Entre os aminoácidos funcionais, a arginina ocupa uma importante posição, pois está envolvida em inúmeras vias metabólicas, além de ser mais abundante carreador de nitrogênio para as proteínas do tecido (Wu, 2013), e vem sendo estudada na nutrição animal.

2.3. Arginina

A arginina (2-amino-5- guanidinovaleric ácido) foi descoberta em 1886 em plantas, e em 1895 foi identificada como parte da proteína animal (Palmer et al., 1988). Desde então, é estudada incessantemente por pesquisadores de diversas áreas que vão desde a saúde humana à produção animal (Tapiero et al., 2002).

A arginina é um aminoácido essencial para peixes, e classificada como versátil, por estar envolvida diretamente ou na forma de derivados em diversos processos

fisiológicos. Como citado anteriormente, inúmeros estudos mostram a relevância deste componente no desempenho zootécnico de peixes (Cheng et al., 2012, Pereira et al., 2017). A exigência de arginina para o *Rhamdia quelen* ainda não é padronizada, e não se sabe seu efeito sobre os processos biológicos e fisiológicos desencadeado durante os eventos reprodutivos.

Abundante em proteínas do tecido (Wu et al. 1999) este aminoácido é popularmente conhecido como o mais versátil em células animais (Wu & Morris 1998). De acordo co Wu (2010) a arginina faz parte de um grupo denominado aminoácidos funcionais, que é definido como aqueles que participam e regulam as principais vias metabólicas e influenciam no crescimento e saúde de mamíferos e peixes. Este importante componente da proteína (Walsh & Mommsen, 2001), é um aminoácido essencial para aves, carnívoros, mamíferos nas fases jovens e peixes (Wu 2010). E atua como precursora da síntese do óxido nítrico, ureia, poliaminas, prolina, glutamate, creatina e agmatina (Wu & Morris, 1998). Além disso, é precursora da creatina e da síntese do oxido nitrico, e serve como estimulador da insulina, glucagon e hormônio de crescimento, prolactina (Wan et al., 2006). Afeta a liberação de hormônios de crescimento (Mommsen, 2001) e aumenta a imunidade (Zhang et al., 2017, Buntello et al, 2007), o que já foi comprovado por Field et al. (2002) e Wu et al. (2004). Sua atuação nos processo fisiológicos ocorre tanto na sua forma direta ou derivados. Ela é um abundante transportador de nitrogênio para proteínas dos tecidos, além de fazer parte da via de regulação de enzimas como arginase, óxido nítrico sintase, entre outras (Luo et al., 2004, Wu et al. 2009, Wu, 2010, 2013a).

A arginina recebe uma atenção especial nas pesquisas principalmente porque é apresenta papel exclusivo na biosíntese das poliaminas (putrescina, espermidina e espermina) Cheng et al., (2011), que por sua vez mostraram o efeito significante no crescimento da mucosa do intestino (Péres et al., 1997), aumentando a proliferação celular e o peso do intestino de ratos (Sukhotnik et al., 2005).

É um aminoácido limitado em plantas utilizadas como fonte proteica, tal como, milho e dietas baseadas em caseina, demandando sua suplementação para atender a necessidade animal (Mai et al., 1994, Wilson, 2002, Singh & Khan, 2007). A exigência deste aminoácido em peixes apresenta um amplo espectro, e varia de 3,0 a 8,1% (NRC, 2011). Na literatura existe uma ampla variação dos níveis recomendados, inclusive para

a mesma espécie, Kim et al. (1992) e Luo et al. (2004) atribuem essa variação ao tamanho do peixe, fonte e níveis de proteína na dieta e condições experimentais.

Este aminoácido vem sendo estudado na aquicultura em pesquisas que avaliam o efeito da sua suplementação no desempenho zootécnico de peixes. Segundo Pholenz et al. (2013), Chen et al. 2012b e Tu et al. (2015) a arginina tem efeito sobre a liberação de fatores de crescimento e insulina (fator de crescimento), sendo que a arginina age ativando a produção e consequentemente, acelera a utilização nutricional. Ao contrário, sua deficiência causa mortalidade e lordose em carpa comum (Tacon, 1992), além de redução do crescimento, eficiência alimentar e da retenção de proteína (Fournier et al., 2003, Wilson, 2002), enquanto a sua suplementação adequada melhora o crescimento, a eficiência alimentar, a imunidade e resulta em efeitos positivos para tilápia do Nilo, *Oreochromis niloticus* (Neu et al., 2016, Pereira et al., 2017), catfish amarelo, *Pelteobagrus fulvidraco* (Chen et al., 2016), linguado, *Scophthalmus maximus* (Zhang et al, 2017), bagre do canal, *Ichtalurus punctatus* (Pohlenz et al., 2013), striped bass hibrido (Cheng et al., 2012).

2.4.Arginina na reprodução

Outra característica da arginina na produção animal é seu efeito positivo sobre a reprodução, que já foi comprovado em várias espécies, entretanto, não se conhece seu efeito sobre a reprodução de peixes. A versatilidade da arginina a torna um importante componente também em estudos reprodutivos humano e animal. Em 1924 este aminoácido foi verificado como o mais abundante entre a proteínas básicas no sêmen de peixes. A sua deficiência na dieta de homens pode causar perda da motilidade espermatica e além de uma diminuição expressiva na quantidade de espermatozoides (Wu et al., 2008).

A ação da arginina sobre a reprodução de mamíferos é muito estudada, e o seu papel tem efeito comprovado nos gametas masculinos, femininos e a prole. A arginina desempenha papel estimulante na motilidade espermática de humanos, coelhos e cabras (Aydin et al., 1995, Radany et al., 1981, Patel et al., 1998, Srivastava et al., 2006), e sua deficiência na dieta pode causar perda da motilidade espermática em homens, além de diminuir expressivamente a quantidade de espermatozoides (Wu et al., 2008). Ainda

prolonga a atividade espermática, viabilidade e integridade da membrana em sêmen bovino criopreservado (Siddique & Atreja, 2013).

Estudos demonstraram que existe correlação positiva entre a suplementação de arginina com número, tamanho e sobrevivência da prole (Wu et al., 2012, McCoard et al., 2013, Zhang et al., 2016a, Zhang et al., 2016b). Por exemplo, em suínos, a suplementação de arginina na gestação aumenta o número de descendentes (Mateo et al., 2007). Além disso, entre o 10° e 16° dia de gestação de ovinos a concentração de arginina, histidina, ornitina e lisina no útero aumentam expressivamente (Gao et al., 2009), reportando à importância crucial destes aminoácidos no crescimento e desenvolvimento da prole (Wu 2009).

Sabe-se então, que a arginina é essencial para a gestação em mamíferos (Wu et al, 2009), embora não seja claro como a arginina beneficia a reprodução sugere-se que sua ação agiogênica, decorrente da produção de óxido nítrico, pode ser o fator chave durante a gestação (Wu et al., 1996), pois aumentam a circulação sanguínea. Greene et al., (2011) verificaram que a suplementação dietética de arginina aumentou o número de ratos viáveis nascidos, demonstrando o efeito benéfico da arginina na reprodução de mamíferos. A arginina ainda é precursora das poliaminas, que são fundamentais para a reprodução, e são sintetizadas nas células de Sertoli e Leydig, além de terem importante papel na implantação dos embriões (Lefreve et al., 2011). Além disso, entre as poliaminas existe a espermina, que é necessária para a completa espermatogênese, sem a qual promove infertilidade em ratos (Pegg et al., 2009).

Todos os resultados encontrados deixam clara a importância da arginina na reprodução, sendo de forma direta ou na forma de derivados. Grande parte desses resultados são relacionados com o fato de arginina ser a precursora do óxido nítrico e, este por sua vez atuar na reprodução devido sua característica versátil e onipresente.

2.5.Óxido nítrico e reprodução

Desde meados dos anos 1990, estudos sobre a síntese de óxido nítrico (NO) passaram a receber cada vez mais atenção (Morris 2000). O NO é, fisiologicamente, um importante gás sinalizador (Li et al., 2009), que estimula a proliferação celular e migração, remodelação celular, angiogênese e dilatação dos vasos para aumento do fluxo sanguíneo (Wu et al. 2009). Estudos afirmam que os peixes podem produzir NO pelo

NOS (Buentello & Gatlin, 1999), bem como ureia e ornitina pela arginase (Gouillou-Coustants et al., 2002). O NO é conhecido como uma molécula sinalizadora universal em células e tecidos de plantas, e atua na resistência de uma variedade de estresse, tais como metais pesados (Kaya & Ashraf, 2015). De acordo com Tapiero et al. (2002) o NO atua como neurotransmissor e mediador das repostas immune, sendo o nitrato seu principal produto final.

Sua síntese ocorre pela conversão de L-arginina em L-citrulina, mediado pela família de isoformas conhecida como óxido nitrico sintase (NOS) (Lind et al., 2017). Em mamíferos o aumento da produção do NO e da vasodilatação ocorre em resposta ao aumento de L-arginina, substrado do NOS (Palmer et al, 1989). Existem três formas de NOS), a neuronal NOS (nNOS, também conhecida como NOS1), indusível NOS (iNOS ou NOS2), e endotelial eNOS (eNOS , ou NOS3). Todas as isoformas são similares no sentido de produzirem NO e outras moléculas biológicas como a L-citrulina (Forstemann, 2012) via utilização da arginina. Todas as isoformas são estudadas em mamíferos desde a sua descoberta, entretanto, apenas no ano de 2011 foram detectadas em peixes, e ainda com dúvidas sobre a real existência de eNOS (Mueller & O'Brien, 2011) e, apenas recentemente, Singh & Lal (2017) constataram todas as isoformas de NOS nos testículos de *Clarias batrachus*.

Existem diversas evidências que sustentam o papel fisiológico do NO na regulação de eventos reprodutivos tais como: a regulação de secreção de gonadotropinas, esteroidogênese, desenvolvimento folicular, ovulação, entre tantos outros (Zamberlam et al., 2014, Falleti et al., 2003). E os estudos que avaliam a suplementação de arginina na dieta de reprodutores atribuem o sucesso dos resultados ao óxido nítrico (Rosseli et al 1998, Wu et al., 2012). O NO é catalizado através da ação de uma das três isoformas da NOS na presença de arginina e NADPH em diversos tecidos, incluindo as gônadas (Singh & Lal, 2015). Atua como molécula sinalizadora em funções reprodutivas tanto em machos quanto em fêmeas. Em mamíferos atua na espermatogênese, ereção peniana e fertilização (Rosseli et al., 1998), além de regular diretamente a espermatogênese nas células de Leydig e modificar a ação de outros hormônios enviados aos testículos. Em fêmeas, influencia na esteroidogênese ovariana, desenvolvimento folicular, ovulação, qualidade dos ovocitos e atresia (Rosseli et al., 1998, Goud et al., 2005, Mitchel et al., 2004, Tamanini et al., 2006).

Além de controlar a produção hormonal o NOS controla o comportamento reprodutivo (Pazinca et al, 2006). Dixit & Parvizi (2001) mostratam em uma revisão que existem inúmeras evidências sobre a ação do NO sobre o eixo hipotálamo, pituitária, gônadas, onde é possível observar que este gás age sobre a pituitária e aumenta o LH e FSH, age ainda no hipotálamo promovendo o aumento do GnRH e diretamente no ovário onde aumenta a progesterona. Na mesma revisão, Dixit & Parvizi (2001) mostram que as três isoformas foram identificadas no sistema reprodutivo feminino, em mamíferos, onde o NO atua em uma grande variedade de eventos reprodutivos, incluindo desenvolvimento folicular ovulação, esteroidogênese, maturação, entre outras.

Estudos recentes tem mostrado a ação do NO na reprodução de peixes, apesar da limitação no número de trabalhos e espécies estudadas, os resultados são extremamente interessantes e encorajadores. Recentemente, foi verificada a expressão de nNOS e iNOS nos testículos de *Clarias batrachus*, sugerindo que o NO é modulador da androgênese em peixes, e que excerce papel regulatório durante a espermatogênese (Lal & Dubey, 2013). Singh & Lal (2017) encontraram pela primeira vez todas as isoformas do NOS em diferentes tipos de células intersticiais, bem como, nas células de Sertoli, e estabelecem que o NO atua como um importante modulador da secreção de esteroides e da espermatogênese. Em fêmeas de *Clarias batrachus* Singh & Lal (2015) encontraram todas as isoformas do NOS nos ovários de peixes, principalmente nos folículos em desenvolvimento (oócitos II e III), e sugeriram que o NO atua diretamente na foliculogênese e esteroidogênese.

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3. DOUTORADO SANDUÍCHE

No ano de 2017, fui contemplada com uma bolsa de doutorado sanduíche para estudar seis meses na França. O estágio foi realiado no INRA – "Instituto Nacional de Pesquisa Agronômica" na unidade LPGP "Laboratório de Fisiologia e Genômica de Peixes" sob surprevisão do Dr. Julien Bobe.

Devido à escasses de tempo ábil foi impossível realizar análises do material obtido nos experimentos do doutorado, e foi então decidido pelo orientador francês realizar um treinamento de análises moleculares para que fossem posteriormente aplicadas na sequência do material da tese e durante o pós doutorado.

Durante o estágio a principal atividade foi referente à um projeto intitulado Hot-Mamma que avalia o efeito de altas temperaturas durante a fase final de maturação na prole de truta arco íris - *Oncorhynchus mykiss*. Além disso houve atividade em um experimento com nutrição de reprodutores de truta arco íris que avalia diferentes quantidades de proteína bruta na ração e seu efeito sobre a qualidade dos oócitos, produção hormonal e desenvolvimento da prole.

As análises realizadas foram: extração de mRNA, avaliação da integridade do mRNA, qPCR em tempo real. Para isso houve treinamento com acompanhamento de uma técnica de laboratório durante um mês, posteriormente ao treinamento todo material do Hot-Mamma foi manipulado e analisado por mim. Ao fim do estágio o resultado foi, além do aprendiado sobre as técnicas, um resumo apresentado na Republica Tcheca e um artigo com co-autoria que será publicado. Além das atividades mencionadas semanalmente participei de todas as reuniões do grupo, apresentei resultados da minha tese e os laboratórios brasileiros e acompanhei outros estudantes de pós graduação que

trabalhavam com outras técnicas moleculares aplicadas à reprodução de peies como: Crispr-Cas 9, imuno-histoquímica e cultura celular.

Ao final do estágio o Dr. Julien Bobe me forneceu um documento onde me convida a retornar durante o pós doutorado para a execução de uma fase do futuro projeto que poderá ser realizada inteiramente no INRA-LPGP, a fase consistirá na edição de genes para avaliar o efeito da L-Arginina (objeto de estudo no doutorado e pós doutorado) na reprodução de peixes.

Resultado parcial do trabalho realizado no doutorado sanduíche apresentado no 6º Workshop Internacional da Biologia de Gametas de Peixes:

IMPACT OF MATERNAL EXPOSURE TO HIGH TEMPERATURE ON EGG QUALITY AND SUBSEQUENT OFFSPRING BEHAVIOR

Danielle Zanerato Damasceno [1,2*], Morgane Cousture [1], Claudiane Valotaire[1], Aurélie Le Cam[1], Thao Vi Nguyen[1], Julien Bobe[1], Violaine Colson[1]

[1]INRA LPGP, UR1037 Fish Physiology and Genomics, F-35000 Rennes, France; dzdamasceno@gmail.com

[2]CAUNESP, Aquaculture Center of São Paulo State University, CEP-14884-900, Jaboticabal- SP, Brazil

In the current context of global climate warming, aquaculture fish are exposed to varying environmental factors including suboptimal temperatures at specific periods of their lifecycle. Fish are highly sensitive to extreme or abnormal (i.e. outside of the normal physiological range) temperatures throughout their lifecycle. This is especially true for key periods such as critical steps of gamete formation. The reproductive period, during which the female gamete undergoes final oocyte maturation, is very sensitive to suboptimal temperature exposure, even for short periods of time. The direct impact on gamete – especially the female gamete – quality has been thoroughly investigated in many temperate species. The direct impact on gamete – especially the female gamete – quality has been thoroughly investigated in many temperate species. Despite this well documented negative impact on egg quality and subsequent embryonic development, the long-term effects have been poorly investigated and data on subsequent fish performance and adaptive capacities are scarce. The aim of the study was to investigate the intergenerational consequences of mother exposure to abnormal temperature on offspring behavioral plasticity in rainbow trout (*Oncorhynchus mykiss*). Sixty females at end of oogenesis were kept in normal (12°C) and high (17°C) water temperature. Fish were

checked for ovulation every 2-3 days. Eggs (approximately 500 per female) were fertilized with a pool of sperms and incubated at 10°C for 138 days after fertilization. Developmental success was monitored at 3 stages: eyed stage (18 dpf), hatching (33 dpf) and yolk-sac resorption (70 dpf). In each phase one sample of embryos was frozen in -80°C for molecular analysis. Between 75 and 138 dpf behavioral phenotyping was performed using tests designed to evaluate fear and learning performance. For fear we used an open-field test, a sudden event test and an emergence test. For learning we used a spatial learning task with a T-maze. Developmental success was significantly lower in embryos originating from females exposed to 17°C, in comparison to embryos originating from females held at 12°C. An overall developmental success of 86% was monitored in the control (females held at 12°C) group while it was only 47% in the eggs originating from females exposed to high temperature (17°C). The behavior performance showed that progeny of 17°C fish presented a phenotype towards more proactive profile but with a slower learning than the control group. This shows that the thermal stress during late oogenesis triggers an increase in embryonic mortality and differences in behavior in the progeny. Molecular analyses are currently in progress to understand the molecular mechanisms mediating the intergenerational impact of maternal exposure to high temperature on egg quality and offspring adaptive capacities.

Key-words: fish reproduction, rainbow trout, global climate warming, oocyte, fish larvae.

ARTIGO I

Este artigo é parte da tese intitulada “Arginina para reprodutores de *Rhamdia quelen*” e está formatado de acordo com as normas da revista científica Animal Reproduction Science, https://www.journals.elsevier.com/animal-reproduction-science/

4. Artigo I

4.1. Arginine influences the reproductive parameters of *Rhamdia quelen* males

HIGHLIGHTS

-Arginine has effect on reproduction in *Rhamdia quelen* males;

-Addition of 2.27% arginine increased seminal volume and sperm concentration;

-Concentration of nitric oxide increases as the percentage of arginine in the diet grows and interferes in sperm motility,

-In tissue culture the nitric oxide concentration increases with arginine presence.

ABSTRACT

Arginine has been studied in the nutrition of mammal breeders, but this is a pioneering study for fish. With the objective of assessing the effect of arginine on the reproductive parameters of *R. quelen* males, five diets were formulated with different levels of arginine (1.37; 1.67; 1.97; 2.27 and 2.57%). After five months of experiment the fish received hormonal induction (2,5 $mg.kg^{-1}$ carp pituitary extract), the semen were collected and analysed: volum, pH, concentration, velocity, motility and normality. The fish were euthanazied, the liver, visceral fat and testis were collected to somatic analysis. In a second moment, were performed the tissue culture with different arginine concentration in medium (0; 5 and 10 mM) in 18 hours to quantify the nitric oxide production and seven days to observe the cell proliferation. We have observed that the

addition of 2.27% arginine in the feed increased seminal volume, sperm concentration, nitric oxide and size of testes in this species, although these results have been contrary to those found for sperm motility and velocity. At a later stage, we could observe that the nitric oxide produced was proportional to the arginine added to the medium. We concluded that 2.27% arginine in the diet of *R. quelen* males improve the production and quality of gametes, also increases nitric oxide concentration. Thus, arginine might be a key factor in broodstock nutrition in general.

KEY-WORDS: nutrition, semen, amino acids, nitric oxide, cell culture.

4.1.1. INTRODUCTION

Silver catfish, *Rhamdia quelen* is a species of Siluriformes with wide distribution from the South of Mexico to Argentina (Ittzés et al. 2015). It presents weight gain and growth even at low temperatures (Fracalossi et al. 2004); and another characteristic that favors its production and research is the easy response to artificial reproduction management.

Studies on broodstock nutrition have become increasingly popular in the last few years, and are fundamental to favor gamete development. Studies on *Rhamdia* broodstock nutrition were described by Parra et al. (2010), Reidel et al. (2010), Coldebella et al. (2011) and Tessaro et al. (2012), assessing the seminal parameters and the stages of gonadal development when fish were fed with experimental diets containing distinct levels of crude protein, digestible energy and lipids. However, only Diemer et al. (2014) studied the effects of lysine addition for broodstock in this species.

The importance of amino acids in the diet for primary maintenance of vertebrates is well-known (Wu et al. 2014). The role of arginine as a functional amino acid (Wu, 2009) that acts on the necessary metabolic pathways for the maintenance, growth,

reproduction and immunity of vertebrates (Suenaga et al. 2008) has to be emphasized. Arginine, besides being a constituent of proteins, is also involved in the synthesis of polyamines and proline (Nikolic et al. 2007), as substrate for collagen synthesis and nitric oxide, considered a key component of the seminal plasma and spermatozoa (Wu et al. 2008, Lahnsteiner, 2009). Studies with humans have demonstrated that the addition of micronutrients, including arginine, promotes an increase in seminal volume, concentration and sperm normality (Imhot et al. 2012). According to Scibona et al. (1994), the addition of arginine increases sperm motility in infertile men. Arginine stands out as one of the essential amino acids for fish (Kuçukbay et al. 2008, Wu, 2009, Wu et al. 2014). However, its effects on reproduction is still unknown. In view of that, our objective was to assess the effects of arginine on the reproductive parameters of *Rhamdia quelen* males.

3.1.2 MATERIAL AND METHODS

General information

The experiment was carried out in the Itaipu Binacional reservoir, Foz do Iguaçu, PR/BR (25°26'49" S; 54°32'58" W), for nine months. Eight hundred (800) sexually immature *Rhamdia quelen* juveniles (approximately 25.00 g; 15.00 cm) derived from a commercial fish farm in Toledo/PR-Brazil were used. The fish were distributed in twenty $1m^3$ (1m x 1m x 1m) floating net cages arranged in the reservoir, and fed with experimental feed during the whole period of assessment. The fish were fed four times a day to apparent satiation. This experiment was conducted with the approval of the Animal Experimentation Ethics Committee of the Western Paraná State University, n°04/16.

Experimental design

A completely randomized experimental design was used in the present experiment. It was composed of five levels of arginine (1.37; 1.67; 1.97; 2.27 and 2.57%), and four replicates, in a total of 20 experimental units. One net cage (1m³) with 40 fish was considered as one experimental unit.

Experimental diets

The experimental diets were formulated with five levels of arginine (Table 1), according to the NRC (National Research Council) (2011). The feed contained 35% crude protein (CP) and 3,250 kcal.kg^{-1} digestible energy. The raw material was weighed, homogenized and ground to 0.7 mm particle size, in a Vieira® knife mill, then homogenized again, taken to extrusion (4 mm pellets) in a EXTEEC® machine, and dried in a forced ventilation oven at 55°C for 24 hours. This diet was offered during the whole experimental period.

Table 1. Percent and chemical composition of experimental feed with different levels of arginine.

	Arginine levels (%)				
Ingredients (%)	**T1 1.37**	**T2 1.67**	**T3 1.97**	**T4 2.27**	**T5 2.57**
Corn grain	40.85	41.27	41.69	42.11	42.53
Corn gluten 60%	31.73	31.19	30.65	30.11	29.57
Fish meal	16.00	16.00	16.00	16.00	16.00
Broken rice	5.00	5.00	5.00	5.00	5.00
L-alanine	1.50	1.19	0.88	0.56	0.25
Dicalcium phosphate	1.50	1.51	1.52	1.53	1.53
L-arginine	0.00	0.31	0.63	0.94	1.26
L-lisine	1.05	1.05	1.06	1.06	1.07
Limestone	0.30	0.29	0.28	0.27	0.26

L-treonine	0.62	0.63	0.64	0.65	0.66
Premix*	0.50	0.50	0.50	0.50	0.50
Salt	0.30	0.30	0.30	0.30	0.30
L-tryptophan	0.16	0.16	0.16	0.17	0.17
DL-metionine	0.18	0.19	0.21	0.22	0.23
Choline chloride	0.10	0.10	0.10	0.10	0.10
Vitamin C	0.10	0.10	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10	0.10	0.10
BHT	0.02	0.02	0.02	0.02	0.02
Soybean oil	0.00	0.09	0.17	0.26	0.35
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nutrients (%)					
Starch	34.33	34.33	34.33	34.33	35.04
Total Arginine	1.37	1.67	1.97	2.27	2.57
Calcium	1.44	1.44	1.44	1.44	1.44
DE *R. quelen*	3250.00	3250.00	3250.00	3250.00	3250.00
Fiber	1.09	1.09	1.09	1.09	1.09
Total phosphorus	1.00	1.00	1.00	1.00	1.00
Fat	4.68	4.68	4.68	4.68	5.03
Total Lysine	1.79	1.79	1.79	1.79	1.79
Methyonine+Cystine	1.51	1.51	1.51	1.51	1.51
Crude protein	35.00	35.00	35.00	35.00	35.00
Total Threonine	1.79	1.79	1.79	1.79	1.79
Total Tryptophan	0.35	0.35	0.35	0.35	0.35

*The vitamin and mineral supplement provided the following nutrients in 1 kg of product: vit. A =2 000 000 IU; vit. D3=400 000 IU; vit.E=30 000 mg; vit. K3=2000 mg; vit. B1=4000 mg; vit. B2=4000mg; vit. B6=3000.00 mg; vit. B12=80 mg; folic acid=1000 mg; calcium pantothenate=10 000 mg; vit. C=60 000 mg; biotin=200 mg; choline=100 000 mg; niacin=20 000 mg; iron =16 000 mg; copper=2000 mg; manganese=6000 mg; iodine = 200 mg; and cobalt=60 mg. ** $kcal.kg^{-1}$

Water quality

The parameters of reservoir water quality were monitored daily (08:00 am and 02:30 pm) with a portable Horiba® multiparameter meter model U50. The values verified were: 27.35±2.05°C, 7.70±0.23 pH, 7.74±0.84 $mg.L^{-1}$ dissolved oxygen and 5.71±3.37 NTU turbidity.

Selection of broodstock, semen sampling and analysis

During the breeding season (November to February), 15 mature males (124.93±54.04g; 22.23±2.97cm) were selected per treatment in each month of this period, directly from the net cages. The males were considered mature when released a small amount of semen upon slight abdominal pressure. The fish from each treatment were then taken to the laboratory of reproduction in Canal da Piracema/Itaipu Binacional/ Foz do Iguaçu-Brazil, where they were kept in hapas (0.5 x 0.5 x 0.5m) arranged inside a concrete tank with constant water renewal. The water used in the laboratory tank came from the reservoir.

Each fish received one single intramuscular injection of carp pituitary extract (CPE) into the dorsal region – 2.5 $mg.kg^{-1}$ CPE, diluted in saline (0.6% NaCl). The semen was collected 240 accumulated thermal units after hormonal induction.

The semen was collected in 10 mL (±0.5mL) graded Falcon tubes for volume measurement, and kept in a styrofoam box with ice (Sanches et al. 2013) until the sperm analysis had been conducted:

-pH: measured by the colorimetric method using Merk® litmus paper (Asturiano et al. 2001);

-Sperm concentration (x10^9 spermatozoa.mL^{-1}): measured from the dilution of semen in buffered formol saline (1:1000) and counted by means of a Neubauer hematimetric chamber (Sanches et al. 2011);

-Sperm motility (%) and **Sperm Velocities** (μm.s^{-1}): were analyzed by free software ImageJ (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/) with the application CASA (*Computer Assisted Sperm Analysis* - University of California and Howard Hughes Medical Institute, USA, http://rsbweb.nih.gov/ij/plugins/casa.html). The semen was activated with distilled water (1μL semen: 400 μL water), and assessed under light microscope (obj.10x). Values of motility were obtained simultaneously to the values of curvilinear, average path and straight line velocities of the spermatozoa ten seconds after activation during one second of movement. The videos were recorded (Proscilica® camera model GE680) using 100 FPS frame rate at 640x480 pixels. The videos were processed based on the description of the components required to use the application CASA by means of the free software ImageJ as specified by Wilson-Leedy and Ingeinmann (2007), using the configurations adapted to the species (Sanches et al. 2010).

-Sperm normality (%): 500μL of semen were fixed in buffered formol saline (1:1000 semen:fixative), and then stained with rose bengal (Tessaro et al. 2012). Afterwards, two 10 μL drops of the fixed solution were placed on the end of the glass slide, which was inclined so that the drops could run to the other end of the slide. After the slides had dried in the open air, they were analyzed under light microscope (obj. 40x) (Caneppele et al. 2015). Three hundred spermatozoa from each fish were counted and classified into normal and abnormal according to CBRA (1998).

-Osmolality (mOSM kg^{-1}): The osmolality of seminal plasma was assessed by a PZL® Osmometer model PZL 1000. The values of seminal plasma were assessed after centrifugation of semen for 15 minutes at 3000 rpm.

Organ sampling

The fish were euthanized with benzocaine solution (250.0 $mg.L^{-1}$) according to Resolution number 876 of the Brazilian Council of Veterinary Medicine (2008), so that their organs (testes, liver and visceral fat) could be removed. The tissues were weighed for the calculation of gonadosomatic index (GSI = weight of the testes/total weight of the fish X 100), hepatosomatic index (HSI = weight of the liver/total weight of the fish X 100) and visceral fat index (VGI = weight of viscera/total weight of the fish X 100).

Testis tissue culture

The direct effects of arginine in the *R. quelen* testes was assessed by testis explants, according to the adapted methodology (Leal et al., 2009). Three males in sexual recrudescence were used. They were euthanized and had their testes dissected out. The experiment was conducted in two phases: 18 hours (assessment of NO production and percentage of spermatogenic cysts) and seven days (cell proliferation). The testes were fragmented, weighed and placed on a nitrocellulose membrane on top of an agar block. Testis fragments were then incubated in Leibovitwz L-15 medium (Sigma®) with the addition of arginine (5 and 10 mM) or not, 12 replications of each animal were used for each concentration of arginine and time of culture. In order to evaluate cell proliferation, BrdU (100 µg/ml; 5-bromo-2-deoxyuridine; Sigma Aldrich®) was added to the medium in the last six hours of incubation. Both cultures were carried out at 28ºC in a humid environment (for the second one, the medium was changed every two days). After the period of culture, the gonads were fixed for two hours at room temperature in methacarn

[(v/v) 60% absolute alcohol, 30% chloroformium and 10% glacial acetic acid]. Next, the material was dehydrated in alcohol, included in historesin (Technovit 7100 - Heraeus Kulzer, Wehrheim, Germany), sectioned 4 µm thick and submitted to BrdU immunodetection (Leal et al. 2009).

The 18-hour culture medium was frozen for further measurement of NO concentration. The percentage of spermatogenic cysts was obtained by the total count of cysts present in 50 fields analyzed for each replica. The index of BrdU incorporation was determined by counting the number of positive BrdU cells among the total total number of cells. The following cell types were analysed: type Aundifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}) and type B spermatogonia, in 50 fields for each replica. The germ cell cysts were identified according to well-established morphological criteria, such as nuclear size, proportion between euchromatin and heterochromatin and number of germ cells per cyst (Leal et al. 2009, Schulz et al. 2010).

Immunodetection of BrdU

Immunodetection of BrdU was performed as previously (Nóbrega et al. 2010). The slides were hydrated in distilled water, incubated for 30 min at 60°C in a 1.0% periodic acid in distilled water. Subsequently, endogenous peroxidase was blocked with 1% hydrogen peroxide in PBS (phosphate buffered saline) (pH 7.4). The non-specific sites were blocked by 5% goat serum diluted in a solution of 1% BSA (bovine serum albumin). The material was incubated for two hours with anti-BrdU rat monoclonal primary antibody (Beckton and Dickinson, Mountain View, CA) diluted at 1:80 in the same blocking solution. After being washed with PBS, the material was incubated in universal secondary antibody (EasyLink One – One Step Polymer HRP) at room temperature for 30 min. Immunodetection was done with DAB (3.3-diaminobenzidine tetrachloride) (Sigma) containing 0.01% hydrogen peroxide in PBS buffer. Histological

sections were counter-stained with Harris hematoxylin and analyzed under conventional light microscope.

Concentration of nitric oxide

The concentration of nitric oxide (NO) was determined in the testes, as described by Panis et al. (2011). The testes were crushed in PBS saline solution at a concentration of 100 mg dry weight of tissue per ml of saline. Tissue suspension was centrifuged nd the supernatant collected. Then, aliquots of 60 µL were deproteinized with 50 µL ZnSO4 75mM, mixture, centrifuged for two minutes and 70 µL NaOH (55mM) was added to the final volume. The samples were again centrifuged for two minutes and the supernatant recovered and diluted in glycine buffer solution (45 g/L pH 9.7) at 5:1 ratio. Cadmium beads kept in H_2SO_4 100 mM solution were washed three times with distilled water and immersed in $CuSO_4$ (5mM) solution in glycine buffer-NaOH (15 m/L, pH 9.7) for five minutes. The treatment with cadmium converts all nitrate into nitrite, providing more accurate results of NO total concentration in the sample. Activated cadmium beads were added to the buffered supernatant with glycine and gently agitated for 10 minutes. Next, 50 µL from each sample was added in duplicate into a 96-well plate. In order to determine the concentration of nitrite in the samples, a standard curve was prepared with $NaNO_2$ dilutions ranging from 250 – 0 µM. Griess reagent was prepared with the addition of the same volume of reagent I (50 mg of N-naphthylethylenediamine in 250 mL of distilled water) and reagent II (5 g of Sulfanilamide in 500 mL of 3M HCl). 50 µL of Griess reagent was added to the plate in 1:1 ratio between Griess reagent and sample/calibration curve. Absorbance was determined at 550 nm using a Biotek, Gen5 microplate reader (Winooski, VT, USA).

Statistical analysis

The values obtained were submitted to the assumptions (tests for normality of residuals -Shapiro-Wilk- and homoscedasticity of variances), and then analysis of variance (*one-way* ANOVA) at 5% significance level, and when significant, the Tukey test of comparison of means (5%) was applied. In order to evaluate correlation between seminal parameters, the Pearson test of linear correlation was applied to the level of 5% significance. The analysis was carried out with the software Statistica 7.1.

3.1.3. RESULTS

Fish fed with 2.27% arginine exhibited the highest values ($p<0.05$) of seminal volume and sperm concentration in comparison to the other levels tested (Table 2). The lowest values ($p<0.05$) for motility (Table 2), as well as velocities (Fig. 1) were found in the same treatment. On the other hand, the normality was higher in treatments between 1.37 and 2.27% of arginine and decreased in the higher concentration treatment ($p<0.05$). The means of seminal pH did not differ ($p>0.05$) between treatments, neither did the values of osmolality of seminal plasma (Table 2). The high sperm concentration of the males fed 2.27% arginine compensated for the lower sperm motility, and resulted in a larger amount of released motile spermatozoa ($p<0.05$) (Table 2).

Table 2. Seminal parameters (mean±standard deviation) of *Rhamdia quelen* males fed diets with different levels of arginine.

Seminal parameters	Arginine levels (%)					
	T1 1.37	**T2 1.67**	**T3 1.97**	**T4 2.27**	**T5 2.57**	***P***
Volume	27.34±14.11^{b}	40.43±6.91^{ab}	39.07±10.91^{ab}	42.58±12.61^{a}	30.01±10.19^{ab}	0.01
Concentration	16.26±6.74^{b}	15.18±7.60^{b}	16.21±4.21^{b}	30.03±17.1^{a}	13.23±1.13^{b}	0.04
pH	8.18±0.24	8.35±0.55	8.25±0.46	8.37±0.35	8.56±0.32	Ns
Normality	77.12±8.43^{a}	74.74±11.37^{a}	75.79±9.83^{a}	76.47±9.45^{a}	56.78±12.40^{b}	0.04
Motility	87.22±5.95^{a}	81.71±5.92^{ab}	84.05±7.89^{ab}	75.22±13.97^{b}	85.87±8.68^{ab}	0.04
MSR	12.01±4.17^{ab}	11.53±4.45^{b}	13.72±4.56^{ab}	26.35±11.76^{a}	11.36±1.06^{b}	0.02
Osmolality	240.11±17.33	221.25±31.03	239.55±26.37	227.90±27.96	241.00±11.61	Ns

Volume: seminal volume released (mL.kg^{-1} of fish); Concentration: Sperm concentration (x10^{9} spermatozoa.mL^{-1}); Normality: sperm normality (%); Motitily: sperm motility %;), MSR: motile spermatozoa released (x10^{9} spermatozoa.mL^{-1}); Osmolality: mOSM kg^{-1}.

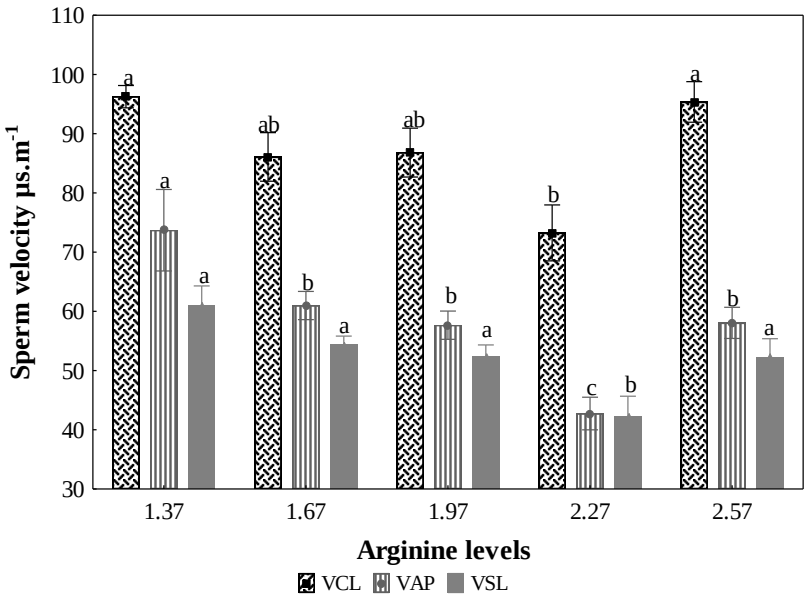

Figure 1. Curvilinear velocity (VCL), Average path velocity (VAP) and Straight line velocity (VSL) of the spermatozoa from *Rhamdia quelen* fed with different levels of arginine. 1: 1.37%; 2: 1.67%; 3: 1.97%; 4: 2.27% and 5: 2.57% arginine. Lower case letters show significant difference among treatments ($p<0.05$).

Interestingly, fish fed with 2.27% arginine presented larger ($p<0.05$) amounts of nitric oxide (NO) in the testes in comparison with other levels (Fig 2). The indexes HSI and VGI did not differ ($P>0.05$) among treatments; however, the lowest gonadosomatic index (GSI) ($p<0.05$) was found in the fish fed with 2.57% arginine (Fig 3).

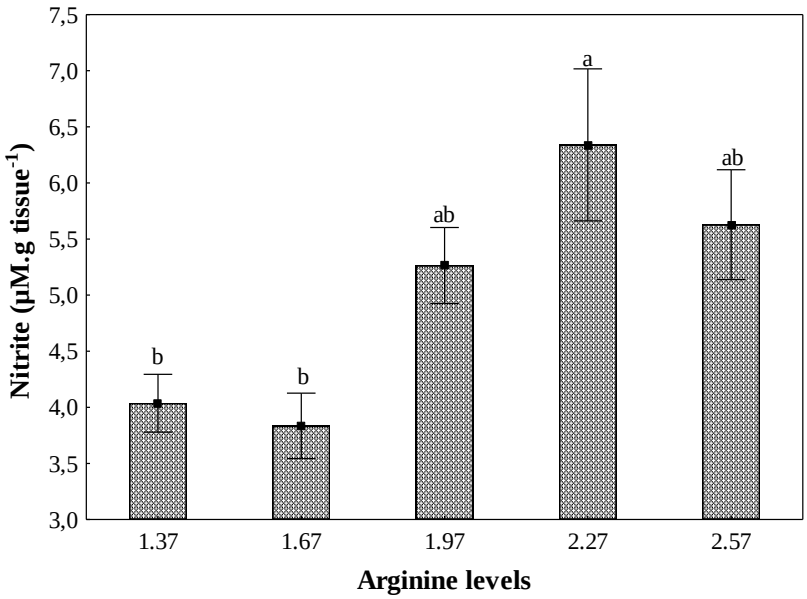

Figure 2. Nitric oxide represented by the amount of nitrite (µM) present in the testes of *Rhamdia quelen* fed with different levels of arginine. The letters show significant difference among the different levels ($p<0.05$).

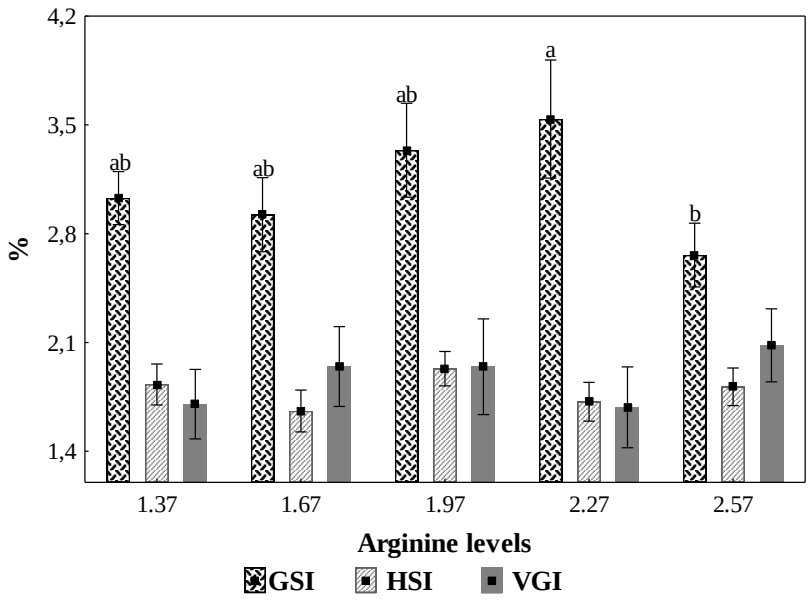

Figure 3. Reproductive indexes (%) of *Rhamdia quelen* males fed with different levels of arginine. The letters represent significant difference among treatments ($p<0.05$). No letters above the bars indicate equivalence statistically. GSI: gonadosomatic index, HSI: hepatosomatic index, VGI: visceral fat index.

Pearson's correlation showed a strong positive correlation between volume and curvilinear velocity (Table 3), and also between motility and all the sperm velocities assessed, which means that when the released seminal volume was high, the spermatozoa exhibited higher curvilinear velocity. Moreover, the results showed that sperm motility has directly influenced the velocities.

On the other hand, nitric oxide presented strong negative correlation with motility and all sperm velocities, suggesting that high amounts of nitric oxide in the testes decrease motility and sperm velocities.

Table 3. Pearson's linear correlation of the seminal parameters of *Rhamdia quelen* fed different levels of arginine.

	Correlation of seminal parameters								
	Volume	**pH**	**Normality**	**Motility**	**VCL**	**VAP**	**VSL**	**Conc**	**NO**
Volume	1,00								
pH	-0,10	1,00							
Normality	0,00	-0,51	1,00						
Motility	-0,46	-0,05	0,37	1,00					
VCL	**-0,68**	0,30	-0,01	**0,78**	1,00				
VAP	-0,15	0,08	0,43	**0,80**	**0,64**	1,00			
VSL	-0,40	0,17	0,29	**0,90**	**0,84**	**0,93**	1,00		
Conc	-0,12	0,00	**-0,66**	-0,32	0,04	-0,45	-0,29	1,00	
NO	0,26	-0,03	-0,30	**-0,87**	**-0,75**	**-0,71**	**-0,80**	0,30	1,00

Volum: volume mL.kg^{-1} of fish; Normal: sperm normality (%); Motil: sperm motility (%); VCL: curvilinear velocity (µm.s^{-1}); VAP: average path velocity (µm.s^{-1}); VSL: straight line velocity (µm.s^{-1}); Conc: sperm concentration (x10^{9} spermatozoa.mL^{-1}); NO: nitric oxide (µM). Values in bold represent significant Pearson's linear correlation ($p<0.05$).

In vitro studies showed a dose-response effect of arginine on the testicular production and release of nitric oxide after 18 hours of culture (Figure 5). Moreover, when investigating the testicular composition, arginine (5mM, 10mM) did not change the frequency of the germ cell cysts after 18 hours of in vitro exposure (Figure 6A). Interestingly, cell proliferation was not affected either after 7 days of culture with different concentrations of arginine (Figure 6B).

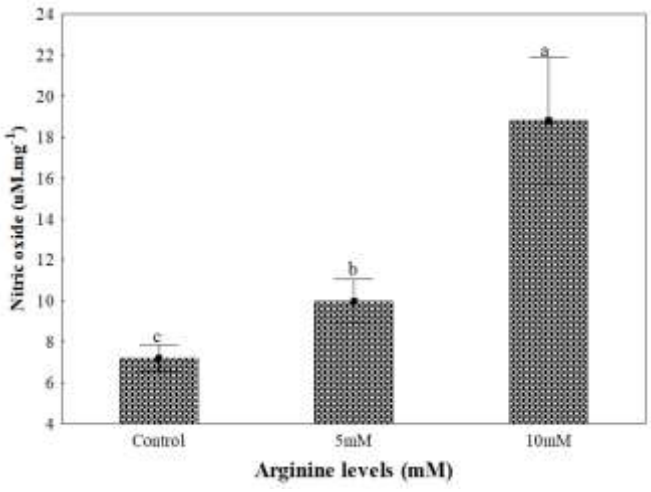

Figure 5. Nitric oxide represented by the amount of nitrite (µM) present in the culture medium after 18 hours, released by one gram of *Rhamdia quelen* testis at different levels of arginine. The letters show significant difference ($p<0.05$).

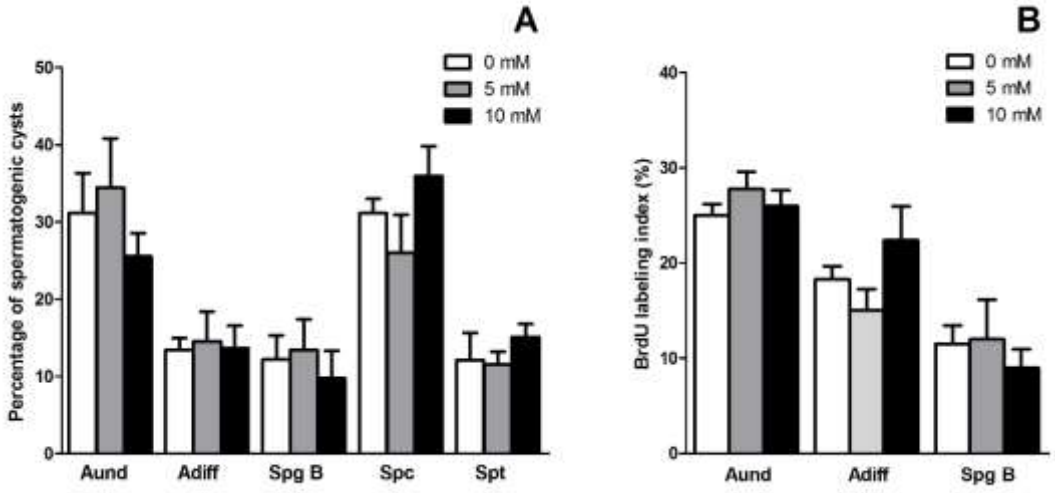

Figure 6. (A) Frequency of cysts of *Rhamdia quelen* testis explants after 18 hours of incubation in the absence (Control 0mM) or presence of 5 or 10 mM arginine. (B) BrdU labeling index for spermatogonia after 7 days of incubation. Results are shown as mean ± standard error. A_{und}= cysts of type A undifferentiated spermatogonia, A_{diff} = cysts of type A differentiated spermatogonia, SPG B = cysts of type B spermatogonia, SPC = cysts of spermatocytes, SPT = cysts of spermatids.

3.1.4. DISCUSSION

In an attempt to verify if arginine influences fish reproduction the same way it influences mammalian reproduction (Balercia et al. 2004, Ren et al. 2015), we have observed that the addition of this amino acid in the diet of *R. quelen* males acted directly on sperm production and seminal parameters. These results reinforce the importance of further studies on the nutrition of broodstock, and the analysis of arginine as well as other amino acids and their action on gamete quality and reproductive success.

The additon of 2.27% arginine increased seminal volume and sperm concentration, making it clear that semen production depends on factors beyond age, size, seasonality and stage of reproductive maturation (Luz et al. 2001, Diemer et al. 2014). Such results have a positive impact on reproduction and optimization of gamete use,

because a lower number of fish can produce enough semen to fertilize a large number of oocytes. These results confirm the ones found by Ren et al. (2015), in which arginine promoted higher sperm concentration and motility in boars. The pH remained within what is considered normal for the species (Table 2), as reported by Tessaro et al. (2012) and Diemer et al. (2014). In fact, Alavi and Cosson (2005) had warned that pH has effect on the activation of fish spermatozoa. However, in this context it is fundamental that the diet does not interfere with the semen pH so that it does not harm activation, motility and potential of fertilization of the spermatozoa.

The results of motility and sperm velocities of fish fed with 2.27% arginine presented similar behavior, but contrary to other parameters assessed. Nonetheless, both motility and velocities were in agreement with the standards for this species, ranging between 70 and 85%, respectively (Tessaro et al. 2012, Sanches et al. 2013). The velocities and motility are correlated, but both are inversely proportional to the amount of NO found in the testes. These variables enable us to evaluate semen quality and relate them to the capacity to fertilize oocytes at the right moment, which is fundamental for production and conservation of fish species (Coward et al. 2002). According to Izquierdo et al. (2001) and Rurangwa et al. (2004), this practice is probably influenced by feeding. The fish fed with 2.27% arginine presented a lower sperm motility rate, but produced the highest sperm concentration which compensated for the lower sperm motility and favored the capacity of fertilization. NO is an important inter and intracellular signaling molecule, and it regulates several reproductive functions, such as spermiogenesis in mammals (Roselli et al. 1998), and steroidogenesis of the Leydig cells in fish (Lal and Dubey, 2013). Studies evaluating NO production in fish gonads are still scarce but Wilson-Leddy and Ingermann (2011) verified NO presence in sperm of *Onchorhyncus mykiss*, and nee Pathak and Lal (2008, 2010) reported the existence of NOS/NO system in fish. A study

carried out by Lal and Dubey (2013) showed that fish testes produce NO during the breeding season, and the expression of NO synthase molecules inside the seminiferous tubules suggests its role in fish spermatogenesis and control the testosterone production.

Sperm velocities have a strong correlation with sperm motility. Belén Herrero et al. (2000) also described the correlation between motility and nitric oxide (NO), showing that NO is capable of modulating cyclic adenosine monophosphate (cAMP). However, in humans, high concentrations of NO showed deleterious effects. The same was described by Balercia et al. (2004), comparing the semen of infertile men (higher concentration of NO, lower velocity) and fertile ones. The work above might serve as foundation for similar studies with fish; however, there is any record in literature so far for fish. Nevertheless, according to Yu et al. (2011), the effects of NO concentration on sperm motility in humans is still controversial, because Weinberg et al. (1995) had stated that high concentrations of NO resulted in decrease in motility and viability of spermatozoa.

The morphological characteristics verified in *R. quelen* testes are common among siluriformes in general. The histomorphological analysis in this study confirmed that all the fish were in their reproductive phase and were similar to the studies conducted with the genus Rhamdia (Diemer et al. 2014, Tessaro et al. 2012). The characteristics were not influenced by the diets offered to the fish.

Our in vitro studies showed that arginine stimulated NO production in a dose-response manner. This result is an direct evidence that *R. quelen* is able to produce NO, confirming that this compound might have a role on reproduction, and that arginine present relation with this production. The NO act directly in regulation of Leydig cell steroidogenesis and is expressed in *Clarias batrachus* testis during recrudescence and mature stages, and suggest that fish testis produce NO during the reproduction, presenting

a regulatory rule in hormonal production (Lal and Dubey 2013), and its role of in spermatogenesis is more evident in mammals (Rosselli et al. 1998). Three isoforms of NOS have been found in Leydig and Sertoli cells, endothelial tissue, spermatogonia and spermatids of humans, swines and rodents (Lee and Chang, 2004). In fish, the studies are still incipients, and recently, three isoforms of NOS have been found in interstitial cells, Sertoli cells, and in the seminiferous tubules of catfish *C. batrachus* (Singh and Lal, 2017). These results encourage further studies to investigate the presence of NOS and its role in the testes of *R. quelen* and other fish species.

3.1.5. CONCLUSION

The addition of 2.27% arginine in the diet of *R. quelen* males improves the production and quality of gametes, increases the size of testes, semen production and sperm concentration. This level of inclusion also increases NO concentration. Such information is extremely relevant, considering the research originality and the responses observed. Thus, arginine might be a key factor in broodstock nutrition.

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4. ARTIGO II

Este artigo é parte da tese intitulada “Arginina para reprodutores de *Rhamdia quelen*” Formatado de acordo com as normas da revista científica Fish Physiology and Biochemistry, https://link.springer.com/journal/10695

4.1. Arginine influences reproductive performance of females and progeny growth in *Rhamdia quelen*

ABSTRACT

Studies have shown that adding arginine to the diet of some species of mammals and birds improves reproductive parameters, but there is no information about its effect on fish reproduction. With the objective of assessing the effect of arginine on the reproduction of *Rhamdia quelen* females, 800 fish were fed diets containing 1.37, 1.67, 1.97, 2.27 and 2.57% arginine for seven months. After this period, they were submitted to artificial reproduction for the assessment of reproductive parameters (gonadosomatic index, quality of oocytes, production of vitellogenin and nitric oxide), and initial development of the progeny. The larvae were fed *Artemia salina* for 10 days, and then initial performance was assessed. We observed that the addition of 2.27% arginine to the diet of *R. quelen* females resulted in larger liver. Furthermore, there was increase in the production of vitellogenin, nitric oxide and oocyte diameter. The newly hatched larvae exhibited larger volume of yolk sac, higher survival and growth rates in the first 10 days of life. The addition of 2.27% arginine to the diet of *R. quelen* females favored reproductive parameters, which suggests that arginine increases the nitric oxide production, and consequently raises reproductive efficiency.

KEY-WORDS: amino acids, larviculture, broodstock nutrition, nitric oxide, vitellogenin.

4.1.2. INTRODUCTION

Jundiá, *Rhamdia quelen* is a species of Siluriformes with wide distribution from the South of Mexico to Argentina (Ittzés et al. 2015). Among the characteristics that favor its production is the easy response to artificial reproduction management.

There are several studies with the genus *Rhamdia* which assess broodstock nutrition (Reidel et al. 2010; Coldebella et al. 2011; Tessaro et al. 2012; Diemer et al. 2014),. However, no results of the addition of arginine to the diet of *R. quelen* have been available so far. It is known that broodstock nutrition is important in providing essential nutrients for the development of gonads, eggs and larvae (Lupatsch et al. 2010), and that an unbalanced nutritional status may limit the number and quality of oocytes (Johnston

et al. 2007; Silva et al. 2008). According to Oliveira et al. (2014), proteins and lipids present in the oocytes are the main components of the diet, used as source of nutrients during the process of embryogenesis, and which affect the number and quality of produced gametes. Thus, adequate broodstock nutrition may serve as basis for producing good quality and quantity of fingerlings, ensuring that market need is met (Al-Feky et al. 2014).

Arginine is an essential amino acid for juvenile animals which experience rapid growth (Wu et al. 2009). For freshwater fish, the urea cycle – a pathway for the synthesis of arginine – is simplified when compared to mammals, so arginine deficiency affects growth and protein retention in fish. Besides, there are studies that show the beneficial effect of arginine on the reproduction of birds (Silva et al. 2012; Sharideh et al. 2016), swines (Mateo et al. 2007) and rats (Zeng et al. 2008).

According to Sharideh et al. (2016), the correct supplementation of essential amino acids requires a fundamental characterization of the metabolic effect on egg production, quality, fertility and hatchability. In spite of countless positive results of arginine in bird and mammal reproduction, so far there have been no studies assessing the effect of this amino acid on fish reproduction. Based on that assertion, the objective of this study was to assess the effect of arginine supplementation on the reproductive performance of *R. quelen* females and their progeny.

4.1.3. MATERIALS AND METHODS

Experimental design

The experiment was carried out in the *Itaipu Binacional* reservoir, in the town of Foz do Iguaçu, Paraná, Brazil (25°26’49” S; 54°32’58” W), between the months of August and March. Eight hundred (800) sexually immature *Rhamdia quelen* juveniles (average 25.00 g; 15.00 cm) obtained from a commercial fish farm in the town of Toledo/PR-Brazil were used. The fish were distributed in twenty 1.0 m^3 (1.0 x 1.0 x 1.0m) floating net cages arranged inside the reservoir, and fed experimental feed four times a day to apparent satiation throughout the whole experimental period.

A completely randomized experimental design, composed of five levels of arginine (1.37; 1.67; 1.97; 2.27 and 2.57%), and four replications was used: a total of 20 experimental units. One net cage (1.0 m^3) with 40 fish was considered as one experimental unit. At this stage of development, there was no evidence of sexual dimorphism; therefore, the sex ratio was not defined per net cage.

This study was conducted according to the rules established by the Animal Experimentation Ethics Committee of the Western Parana State University, registered under number 04/16.

2.3. Experimental diets

The experimental diets were formulated according to NRC (2011), Reidel et al. (2010) and Diemer et al. (2014), and contained 35% crude protein (CP) and 3,250 $kcal.kg^{-1}$ digestible energy (Table 1). The raw material was weighed, homogenized and ground to 0.7 mm particle size, in a Vieira® knife mill, then homogenized again, taken to extrusion (4 mm pellets) in an EXTEEC® machine, and dried in a forced ventilation oven at 55°C for 24 hours.

Table 1. Percent and chemical composition of experimental feed with different levels of arginine.

	Arginine levels (%)				
Ingredients (%)	**1.37**	**1.67**	**1.97**	**2.27**	**2.57**
Corn grain	40.85	41.27	41.69	42.11	42.53
Corn gluten 60%	31.73	31.19	30.65	30.11	29.57
Fish meal	16.00	16.00	16.00	16.00	16.00
Broken rice	5.00	5.00	5.00	5.00	5.00
L-alanine	1.50	1.19	0.88	0.56	0.25
Dicalcium phosphate	1.50	1.51	1.52	1.53	1.53
L-arginine	0.00	0.31	0.63	0.94	1.26
L-lisine	1.05	1.05	1.06	1.06	1.07
Limestone	0.30	0.29	0.28	0.27	0.26

L-treonine	0.62	0.63	0.64	0.65	0.66
Premix*	0.50	0.50	0.50	0.50	0.50
Salt	0.30	0.30	0.30	0.30	0.30
L-tryptophan	0.16	0.16	0.16	0.17	0.17
DL-metionine	0.18	0.19	0.21	0.22	0.23
Choline chloride	0.10	0.10	0.10	0.10	0.10
Vitamin C	0.10	0.10	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10	0.10	0.10
BHT	0.02	0.02	0.02	0.02	0.02
Soybean oil	0.00	0.09	0.17	0.26	0.35
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nutrients (%)					
Starch	34.33	34.33	34.33	34.33	35.04
Total Arginine	1.37	1.67	1.97	2.27	2.57
Calcium	1.44	1.44	1.44	1.44	1.44
DE *R. quelen*	3250.00	3250.00	3250.00	3250.00	3250.00
Fiber	1.09	1.09	1.09	1.09	1.09
Total phosphorus	1.00	1.00	1.00	1.00	1.00
Fat	4.68	4.68	4.68	4.68	5.03
Total Lysine	1.79	1.79	1.79	1.79	1.79
Methyonine+Cystine	1.51	1.51	1.51	1.51	1.51
Crude protein	35.00	35.00	35.00	35.00	35.00
Total Threonine	1.79	1.79	1.79	1.79	1.79
Total Tryptophan	0.35	0.35	0.35	0.35	0.35

*The vitamin and mineral supplement provided the following nutrients in 1 kg of product: vit. A =2 000 000 IU; vit. D3=400 000 IU; vit.E=30 000 mg; vit. K3=2000 mg; vit. B1=4000 mg; vit. B2=4000mg; vit. B6=3000.00 mg; vit. B12=80 mg; folic acid=1000 mg; calcium pantothenate=10 000 mg; vit. C=60 000 mg; biotin=200 mg; choline=100 000 mg; niacin=20 000 mg; iron =16 000 mg; copper=2000 mg; manganese=6000 mg; iodine = 200 mg; and cobalt=60 mg. ** $kcal.kg^{-1}$

2.4. Gamete sampling

One hundred females with mean weight and total length 169.65±60.82g and 24.04±2.48 cm, respectively were used for the study. Twenty females were randomly selected from each treatment, respecting the external morphological characteristics (swollen urogenital papilla and bulging abdomen). The fish were transferred to the laboratory of reproduction of *Itaipu Binacional*, in Foz do Iguaçu, PR, Brazil, kept in hapas (0.5 x 0.5 x 0.5 m) arranged inside a concrete tank with constant water renewal. The water used in the laboratory tank had come from the reservoir. Hormonal induction was carried out with Carp Pituitary Extract – CPE (0.5 $mg.kg^{-1}$ representing a preparatory dose, and then, after 12 hours, 5.0 $mg.kg^{-1}$ CPE). After 240 accumulated thermal units from the second induction, the stripping of females was conducted so that the oocytes could be collected. A small amount of semen was withdrawn under slight pressure on the urogenital papilla, and then the males were hormonally induced (2.5 $mg.kg^{-1}$ CPE). The semen was collected in Falcon tubes (15 mL), kept in a styrofoam box at a temperature of ±12°C (Sanches et al. 2013) until the moment of fertilization.

The oocytes were collected after cephalo-caudal abdominal massage. Fertilization was then conducted using the dry method, and the fertilized eggs were transferred to experimental hatcheries (20 L). Egg development was monitored until larvae hatching.

2.5. Blood and organ sampling

After the oocytes had been collected, the fish were anesthetized with benzocaine solution (75.0 $mg.L^{-1}$), their blood was collected with heparinized syringes, and immediately transferred to Falcon tubes (15.0 mL) containing PMSF protease inhibitor (SIGMA®) and centrifuged at 3000 *g* for 15 minutes. The plasma was immediately transferred to cryotubes and kept in a canister of nitrogen at -196°C.

Subsequently, the fish were euthanized with benzocaine solution (250.0 $mg.L^{-1}$) according to Resolution number 876 of the Brazilian Council of Veterinary Medicine (2008) and dissected, so that their ovaries, liver and visceral fat could be removed. The collected material was weighed for the calculation of reproductive indexes, and then ovary fragments were fixed in Alfac (alcohol 80+formalin+acetic acid) for histological analysis. The gonadosomatic index (GSI), hepatosomatic index (HSI) and visceral fat

index (VFI) were calculated from the weight of the ovaries, liver and visceral fat, respectively (Indexes: weight of the organ/total weight of the fish X 100).

2.6. Quality of female gametes

Before the first hormonal induction, a sample of oocytes was collected per fish by means of a plastic catheter (human urethral probe no.8): 1) fixed in Gilson solution (Simpson 1959) for 30 minutes to determine the size of oocytes (Bittencourt et al. 2012); 2) fixed in Serra solution (Leonardo et al. 2005) to follow the position of the nucleus (central or displaced). An aliquot of released oocytes was fixed in Gilson solution to compare the diameter of oocytes before and after hormonal induction. Oocyte diameter (100 oocytes from each female) was measured by means of a BEL stereomicroscope and BEL View 7 software, where the diameter was calculated by the arithmetic mean of the longest horizontal and vertical axes, and then the means were compared between the treatments.

Twelve hours after fertilization (closure of blastoporus), three egg samples from each experimental unit were withdrawn to estimate Fertilization Rate (FR= number of viable eggs x 100/ total number of eggs), and only the translucent eggs were considered fertilized (Okawara et al. 2015).

2.7. Analysis of vitellogenin

The concentration of vitellogenin in the samples was obtained through the Western-Blot technique (Costa et al. 2010). After the electrophoretic separation of the plasma proteins, determined by the Bradford method (1976), they were reduced and denatured with reducing sample buffer (Tris-HCl 1M pH 6.8, SDS, Glycerol, Bromophenol blue and mercaptoethanol).

The plasma proteins were separated in 4% polyacrylamide mini-gel as stacking gel, and 8% for separation with application of vertical electric field. Molecular mass markers (Rainbow BioRad) were used to monitor the electrophoretic profile of vitellogenin. The proteins were stained with Coomassie Blue (R250) so that the formed bands could be viewed.

2.7.1. Western Blot

The proteins of the polyacrylamide gel separated by SDS-PAGE were transferred to a nitrocellulose membrane (BioRad®: 0.45 µm pore size) containing transfer buffer (25 mM Tris base, 192 mM Glycine, 0.037% SDS, 20% methanol). The running speed was adjusted to 100 V, for 60 minutes. In order to check whether the gel proteins had been transferred to the membrane, it was stained with Ponceau dye (diluted in 1% acetic acid). After the membrane had been washed for dye removal, it was blocked with 5% skimmed milk in TBS-T solution (20mM Tris pH 7.4, 120mM NaCl and 0.05% Tween 20) for 1 hour. The membrane was incubated with *R. quelen* anti-vitellogenin antibody (primary) (Moura-Costa et al. 2010) under constant stirring for 16 hours at 4º C at a dilution of 1:180000 (concentration determined in previous experiments). Later, it was washed with TBST. The membrane was then incubated with rabbit anti-immunoglobulin alkaline phosphatase (Promega) for 1 hour at a dilution of 1:7000. The protein was observed in BCIP/NBT substrate, incubated at room temperature for 1-5 minutes under constant stirring. Plasma from *R. quelen* males kept in the same environment was used to serve as counterproof that the environment did not contain endocrine deregulators that might change the analysis.

2.7.2. Enzyme-Linked Immunosorbent Assay (ELISA)

A competitive ELISA was conducted according to Garnayak et al. (2013), with a few alterations. Purified *R. quelen* VTG (20 ng in 100µl/well) was allowed to adsorb into the wells of an ELISA plate (NUNC MAXISORP, USA) overnight at 4ºC in buffered carbonate-bicarbonate pH 9.6. The plates were washed three times with PBST (PBS pH 7.4 and Tween 0.05%) and were then filled with 200 µl of blocking solution (1% BSA) for 2 hours at room temperature. The plate was washed again and VTG standards (0 at 12.800 ng/ml) and plasma samples at dilution 1: 10,000 were added into duplicate wells at 50 µl. Fifty µl of rabbit anti- *R. quelen* VTG serum was then added at a dilution of 1:2,500 to give a final dilution of 1:5,000. Plates were incubated at 4ºC overnight, after which they were washed as before. Each well then received 100 µl of goat anti rabbit Ig G horseradish peroxidase (HRP) conjugate dextran-HRP diluted (1:4000) in PBST and incubated for 1 hour at room temperature. Subsequently, it was washed three times and the wells received 100 µl of chromogen substrate (5 ml of citrate buffered 0.1M pH 5.0, o-Phenylenediamine (5 mg of OPD and 5 µl of 30 % H_2O_2). Color development was allowed for 15 minutes at room temperature in the dark, and the reaction was stopped by

adding 30 µl of 1 M H2SO4. Absorbance values were measured at 490 nm with an ELISA plate reader.

2.8. Concentration of nitric oxide

The concentration of nitric oxide (NO) was determined in the ovaries as described by Panis (2011). The tissue was crushed in PBS saline solution at a concentration of 100 mg dry weight of tissue per milliliter of saline. Aliquots of 60.0 µL supernatant were deproteinized with 50.0 µL ZnSO4 75mM, followed by vortexing and centrifugation at 5,600 g for 2 minutes, at 25ºC. 70.0 µ L NaOH (55mM) were then added. The samples were vortexed and centrifuged again at 5,600 g for 2 minutes, at 25ºC. The final supernatant was recovered and diluted in glycine buffer solution (45 g/L pH 9.7) at a 5:1 ratio. Cadmium beads kept in H_2SO_4 100 mM solution were washed three times with distilled water and immersed in $CuSO_4$ (5mM) solution in glycine buffer-NaOH (15 m/L, pH 9.7) for 5 minutes. Approximately 600-1000 mg of activated cadmium beads were added to the supernatant buffered with glycine and gently stirred for 10 minutes, so that nitrate could be converted into nitrite.

50.0 µL aliquots from each sample were added in duplicate to the wells of a 96-well plate. In order to determine the concentration of nitrite in the samples, a calibration curve was prepared with a dilution of $NaNO_2$ in distilled water, creating concentrations between 250 – 0 µM. Griess reagent was prepared with the addition of the same volume of reagent I (50.0 mg of N-naphthylethylenediamine in 250.0 mL of distilled water) and reagent II (5.0 g of Sulfanilamide in 500.0 mL of 3M HCl). 50.0 µL of Griess reagent was added to the plate at a 1:1 ratio between Griess reagent and sample/calibration curve. Absorbance was determined at 550.0 nm using a Biotek, Gen5 microplate reader (Winooski, VT, USA).

2.9. Larviculture

After hatching, 50 larvae from each treatment were fixed in 10% formalin solution so that the volume of the yolk sac (mm^3) and initial total length (mm) could be measured. As soon as the larvae presented an active gastrointestinal tract (opening of the mouth and

anus), they were transferred and distributed in 20 polyethylene boxes (21 L of usable volume), at a density of 10 larvae.L^{-1}, distributed in a completely randomized experimental design, consisting of five treatments and four replications (5x4), with an individual system of water circulation, where they remained for 10 days.

Magnetic *Artemia salina* INVE® was used for feeding, 10 times per day (05:00 a.m. to 11:00 p.m.). Once a day, the aquariums were siphoned for the removal of leftover food, feces and dead larvae. The water parameters were recorded daily, and at the end of the experiment, the arithmetic mean was obtained: water temperature 24.40^{o} C; pH 6.84; dissolved oxygen 6.33 mg L^{-1}; turbidity 0.5 NTU and electric conductivity 117.0 mS/m^2.

The artemia cysts were hatched every two days in a conical-cylindrical hatchery (30 L) with constant aeration and illumination located at the bottom and on top of the hatchery, respectively. At each meal, the aliquots were estimated by counting in triplicate the nauplii present in 1.0 mL of water (Okawara et al. 2015). At the end of the experimental period (10th day), the larvae fasted for nine hours, and were later counted to determine the survival rates. Sixty larvae from each experimental unit were fixed in buffered formalin so that the following individual parameters could be measured: initial total weight (mg), total length (mm), standard length (mm), head length (mm), body height (mm), and final total weight (g). The larvae were weighed in a precision analytical balance with 0.0001g accuracy, and measured with a BEL-IBD-45B trinocular magnifying glass attached to a digital camera and a laptop containing BEL View7 software.

2.10. Statistical analysis

The data obtained were submitted to tests of normality (Shapiro-Wilk) and homoscedasticity, and subsequent analysis of variance. When the ANOVA was significant for the variable means ($p<0.05$), the Tukey test was applied for the comparison of means. The software Statistica 7.1. was used for the analysis.

4.1.4. Results

The migration of the nucleus observed in the five treatments was higher than 60% and did not differ significantly between treatments. It was observed that *R. quelen* oocyte release was significantly influenced by arginine supplementation, because over 80% of

the females that had received diets containing more than 1.97% arginine spawned (Figure 1).

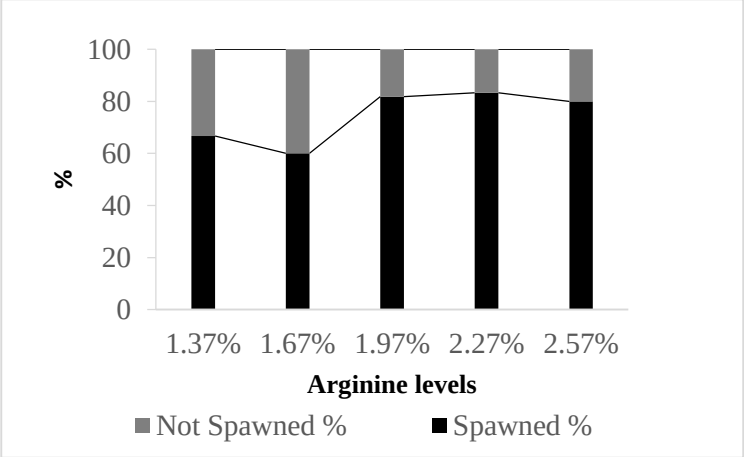

Figure 1**.** Percentage of *Rhamdia quelen* females fed diets with arginine supplementation that spawned after hormonal induction.

The values of fertilization rate were only numerically higher in the treatments with 2.27 and 2.57% arginine (70.78 and 72.54% respectively). However, there were no significant differences (Table 2).

Table 2. Reproductive parameters of females fed diets containing different levels of arginine.

	Reproductive parameters					
	1.37%	**1.67%**	**1.97%**	**2.27%**	**2.57%**	**p**
Fertilization%	60.63±17.35	59.16±5.74	65.51±5.08	70.78±9.17	72.54±16.81	Ns
D. O. B	0.68 ± 0.09^{b}	0.70 ± 0.08^{ab}	0.68 ± 0.09^{b}	0.73 ± 0.09^{a}	0.70 ± 0.09^{ab}	0.03
D. O. A	0.68 ± 0.07^{d}	0.70 ± 0.07^{c}	0.72 ± 0.07^{bc}	0.77 ± 0.05^{a}	0.75 ± 0.07^{ab}	0.04
GSI%	10.24±4.96	10.56±5.28	12.9±3.91	9.47±4.69	8.72±5.53	Ns
HSI%	2.02 ± 0.71^{b}	2.08 ± 0.38^{b}	2.69 ± 0.70^{ab}	2.86 ± 1.02^{a}	2.95 ± 0.65^{a}	0.03
GVI*%	1.62±0.66	2.46±1.29	1.62±0.92	1.71±1.06	1.55±0.61	Ns

D.O. b: Diameter oocyte b (µm) before induction; D.O: Diameter oocyte a (µm) after induction. GSI: Gonadossomatic index; HSI: Hepassomatic index; GVI: índice de Gordura Visceral.

Females fed 2.27% arginine produced oocytes with larger diameter ($p<0.05$), both at the moment of pre-hormonal induction (0.73±0.09 mm) and at the moment of spawning

(0.77±0.05 mm) (Table 2). The mean values of gonadosomatic index did not differ between treatments, but on the other hand, the hepatosomatic index was higher in the treatments with 2.27 and 2.57% arginine (2.86±1.02 and 2.95±0.65%, respectively) (Table 2). The values of GSI and HSI were proportionally inverse.

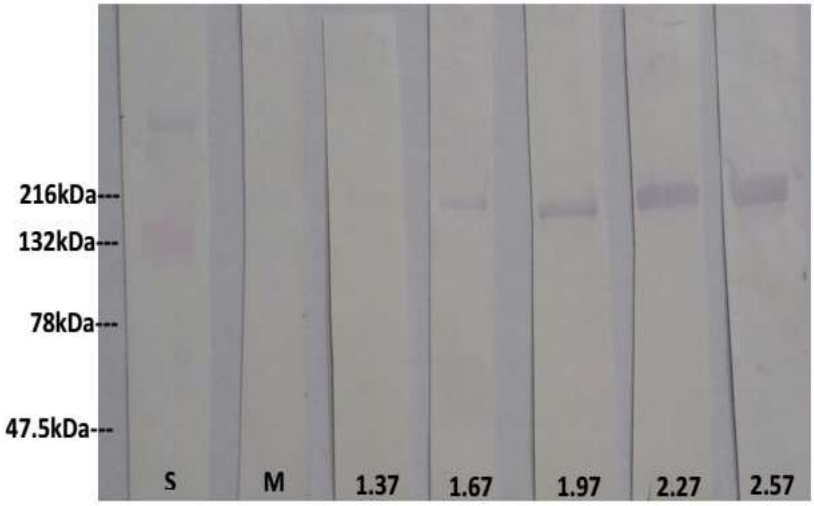

Figure 2. Western Blot technique for plasma vitellogenin in *Rhamdia quelen* females fed different levels of arginine. S: standard; M: male.

NO concentration was higher in the ovaries of females fed diet with 2.27% arginine ($p<0.05$) (Table 3). Meanwhile, we verified that the vitellogenin bands expressed by Western Blot marked clear values in the treatments with 1.97, 2.27 and 2.57% (Figure). The result was confirmed by an ELISA, during which the treatments supplemented with 1.97, 2.27 and 2.57% arginine presented high concentrations of plasma vitellogenin, without statistical differences, though ($p>0.05$).

Table 3. Nitrite found in the ovaries and plasma vitellogenin of *Rhamdia quelen* females fed diets containing different levels of arginine.

	Arginine levels					
	1.37%	**1.67%**	**1.97%**	**2.27%**	**2.57%**	***P***
Nitrite µM	29.81±10.62^{ab}	23.00±7.18^{b}	31.58±9.44^{ab}	38.03±8.57^{a}	33.76±12.40^{ab}	0.01
VTG mg/mL	2.04±0.91	2.02±0.97	2.13±0.90	2.23±1.02	1.87±0.99	Ns

VTG: vitellogenin

The newly hatched larvae (0 to 3 days) from broodstock fed diets containing 2.27 and 2.57% arginine exhibited statistically similar results ($p>0.05$), but differed from the other treatments with regard to volume of yolk sac: 0.23±0.03 and 0.21±0.05mm^3, respectively (Table 4). After the yolk sac had been consumed (4th day after hatching), the larvae from broodstock fed diet with 2.27% arginine exhibited the highest mean of initial total length (4.79±0.27mm) ($p<0.05$). At the end of ten days of larviculture (14 days after hatching), the larvae from broodstock fed diet with 2.27% arginine exhibited the highest results of survival (95.95±0.33%), total length (13.05±1.42mm), standard length (10.77±1.22mm), body height (2.72±0.31mm) and weight gain (0.033±0.013g) ($p<0.05$).

Table 4. Initial performance of larvae from broodtock fed diets with different levels of arginine.

	Larvae quality					
	1.37%	**1.67%**	**1.97%**	**2.27%**	**2.57%**	**p**
Initial lenght (mm)	4.34±0.32^{b}	4.27±0.55^{b}	4.19±0.23^{b}	4.79±0.27^{a}	4.47±0.47^{b}	<0.01
Yolk sac (mm^3)	0.17±0.02^{c}	0.19±0.04^{b}	0.19±0.03^{b}	0.23±0.03^{a}	0.21±0.05^{a}	<0.01
Larvae initial weight (mg)	0.028±0.014^{b}	0.019±0.011^{d}	0.024±0.013^{c}	0.025±0.013^{a}	0.024±0.013^{cd}	<0.01
Final lenght (mm)	12.50±2.07^{b}	11.35±2.12^{c}	11.87±2.01^{c}	13.05±1.42^{a}	10.93±2.80^{c}	<0.01
Body height (mm)	2.73±0.53 ^{a}	2.24±0.45 ^{c}	2.63±0.46 ^{b}	2.72±0.31 ^{a}	2.57±0.77 ^{b}	<0.01
Survival (%)	71.93±2.62^{b}	68.32±11.90^{b}	70.00±8.94^{b}	95.95±0.33^{a}	62.59±4.38^{b}	<0.01

4.1.5. Discussion

Studies that assess fish nutrition and its impact on their progeny are scarce, and the effect of arginine on fish reproduction is still unknown. However, the results found in this study show that the supplementation of this amino acid to the diet of *R. quelen* females improved the reproductive indexes and promoted better initial performance of the progeny.

The addition of arginine at levels equal to or higher than 1.97% promoted higher mean values of HSI and fertilization rate in *R. quelen*, and the diameter of oocytes

obtained from females fed 2.27% arginine was bigger than the other ones. According to Bobe and Labbé (2010), the size and appearance of oocytes may be used to assess their potential for development. The difference in size may be attributed to the nutritional status of the females during ovary development. This is because during ovarian development, both diet and maternal reserves are transported to oocytes to meet the requirements for growth and development, from embryo until the beginning of exogenous feeding (Fontagné-Dicharry et al. 2010; 2017). According to Ghaedi et al. (2016), it is clear that large oocytes produce large larvae, and this higher growth is due to higher amount of yolk, which efficiently nourishes the embryo at the initial phases. In our study, we verified larger volume of yolk sac in larvae obtained from females fed 2.27% arginine. Many nutrients are essential for embryo development, and a proper use of those nutrients through broodstock diet favors the development of normal eggs, as well as the values of hatching rate (Izquierdo et al. 2001). Thus, arginine, with its versatile potential for participating in numerous pathways, proves to be one of those nutrients. Arginine use in fish must be researched more deeply; however, these preliminary results show that there is a relationship between broodstock nutrition and quality of progeny.

In addition to larger diameter and volume of yolk sac, progeny survival was also higher in the treatment with 2.27% arginine, which produced more resistant larvae and with higher potential for growth gain. The influence on vitellogenic reserves (Fernandez-Palacios et al., 2011) might result in permanent effects on the offspring, including growth potential and survival (Fernandez-Palacios et al. 1995; Fontagné-Dicharry et al. 2017). Among the countless metabolic pathways on which arginine acts, there is its participation in the immune system (Wu et al. 2004; Pereira et al. 2017), besides being a potent stimulant of growth hormones (Mommsen 2001) and participating in anabolic processes (Wan et al. 2006). When this amino acid is present in the physiological system, these factors may act concurrently and favor the initial development of larvae, which might cause repercussions in the long term.

In general, studies conducted on broodstock nutrition assess reproductive parameters and oocyte quality, without investigating the quality of the larvae. Nevertheless, the ingredient tested on broodstock might show its effect only on the offspring. Nutritional programming is a study continuously applied in mammals and other vertebrates, which assesses the importance of nutrition since the initial phases of life and its influence during the later stages of development (Patel et al. 2009). In fish, this

procedure has been relatively under-examined, especially when it comes to the effect of female nutrition on progeny performance, but there are important results described by Izquierdo et al. (2015) and Seilez et al. (2017).

Studies have shown that an adequate use of amino acids in nutritional programs of broodstock has effect on their progeny. In the present study, we attested that arginine supplementation showed considerable influence on *R. quelen* larvae. Simultaneously, Fontagné-Dicharry et al. (2017) attested that methionine in the diet of rainbow trout, *Oncorhynchus mykiss* broodstock affects larvae growth, and Seilez et al. (2017) observed that methionine deficiency in the diet of females affects the expression of genes responsible for fundamental metabolic processes in larvae.

We have also observed the highest values of survival rate and initial development in larvae from broodstock that had received diets with 2.27% arginine. The fish that had received that level of arginine also exhibited higher concentration of plasma VTG. Wu et al. (2012) and McCoard et al. (2013) demonstrated a positive correlation between arginine supplementation and number, size and survival of progeny in mammalian species. Arginine has been reported to increase the production of genes connected to reproductive performance (Yao et al. 2011; Liu et al. 2012; Wu et al. 2012), and influence ovarian steroidogenesis, follicle development, ovulation, oocyte quality and follicular atresia (Rosseli et al. 1998; Goud et al. 2005; Mitchel et al. 2004; Tamanini et al. 2006). According to Tandler et al. (1995), broodstock nutrition with an adequate balance of amino acids promotes better synthesis of vitellogenin, and the successful initial development of fish depends on the balance of amino acids present in the eggs (Brooks et al. 1997; Srivastava et al. 1995).

Zhang et al. (2015) claim that VTG and its derivatives present in the yolk exhibit immune, antiviral and antimicrobial activities, being transmitted from the females to their offspring, increasing the progeny's immunity. Arginine has angiogenic action; in other words, it helps blood circulation. That facilitation of blood flow may benefit blood circulation in several organs, including the ovaries, carrying a larger amount of nutrients and VTG to the oocytes. This protein is expressed in the blood plasma of females during vitellogenesis (Hara et al. 2016), is produced in the liver and transported by blood flow to the follicular layer and endocytosed by the growing oocytes, in order to form yolk proteins (Lubzens et al. 2010; Sargent 1995; Wallace 1985). According to Wallace

(1985), it occurs because the estrogen produced by the follicular cells stimulates the synthesis and hepatic secretion of vitellogenin into the systemic circulation.

Vitellogenin is the precursor of egg yolk formation, and is present in female oviparous animals, including fish (Zhang et al. 2015), and as well as in most fish species, it is noticeable during the reproductive phase of *R. quelen*. In fish, it is produced in the liver and transported to the ovaries by the circulatory system; in the ovaries, it acts on oocyte growth (Opresko et al. 1987; Sire et al. 1994). This protein is accumulated in the oocyte and will later serve as a nutrient during embryo development (Arukwe et al. 2003).

We observed that the females from treatment 2.27% arginine exhibited larger amount of NO in the ovaries. The versatile physiological characteristic of arginine makes it a precursor of the synthesis of ornithine, polyamines, proline, glutamine, creatine and NO (Wu et al. 2013). According to Rosselli et al. (1998), the expression of NO in the ovaries depends on the species and also on the stage of maturation of the ovaries. In spite of the few studies assessing NO in fish reproduction, Trikapathi and Krishna (2008), studying *Heteropneustes fossilis* verified that fish ovaries have a nitric oxide synthase – nitric oxide (NOS-NO) system, and that NO may be considered as a mediator in the development of ovarian follicles and a regulator of oocyte maturation. In ovaries of *Clarias batrachus*, all the isoforms of NOS were found, mainly in the developing follicles (oocytes II and III), suggesting that NO acts directly on folliculogenesis and steroidogenesis (Singh and Lal 2015), and induces the secretion of luteinizing hormone (Al-Daraji and Thair 2014), promoting efficient ovulation (Basioni et al. 2006).

As mentioned previously, studies with mammals have demonstrated that there is a positive correlation between arginine supplementation and number, size and survival of progeny (McCoard et al. 2013; Zhang et al. 2016a; Zhang et al. 2016b), associating those good results with NO. In swines, for example, arginine supplementation during pregnancy increases the number of offspring (Mateo et al. 2007). These results are associated with increased blood flow in the placenta, which occurs due to the angiogenic role of nitric oxide (Raghavan and Dikshit 2004), which facilitates blood flow and is a potent promoter of endothelial permeability (Valdes and Corthon 2011). In fish, during the process of vitellogenesis, nutrients such as amino acids are passed from the liver to the oocytes through blood flow (Fontagné-Dicharry et al. 2017). The nutrients are sent to the ovaries, where they will be kept in the oocytes. Although the reproductive physiology of fish is

not exactly the same as the mammals', these results have guided us in the interpretation of the role of NO. However, there are no conclusive studies on its biological mechanism involved in gamete formation.

4.1.6. Conclusion

The present study has shown that with an adequate arginine supplementation, *R. quelen* oocytes and progeny responded positively, favored the reproductive parameters (HSI, oocyte diameter), raised the amount of nitric oxide in the ovaries, as well as promoted higher survival and length for the larvae. These are results that encourage further research to understand the mechanism of action of this amino acid in fish ovaries as well as its relationship with the quality of gametes and progeny. Moreover, it confirms the importance of a sequence of nutritional programs for broodstock.

4.1.7. Acknowledgment

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4.1.8. References

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5. CONCLUSÃO GERAL

Os experimentos conduzidos com arginina na dieta de *Rhamdia quelen*, mostraram pela primeira vez que este aminoácido apresenta papel na reprodução tanto para machos quanto para fêmeas de peixe. Foi verificado que em machos há um aumento do tamanho dos testículos, a produção de sêmen e concentração de espermatozoides. Em fêmeas a suplementação promoveu maior índice hepatossomático, maior diâmetro dos ovócitos, e consequentemente gerou larvas maiores e mais resistentes, as larvas apresentaram sobrevivência notavelmente superior. Em ambos os sexos a arginina influenciou na produção de NO nas gônadas.

O ponto forte deste trabalho, além do ineditismo do tema, é a confirmação de que a arginina desempenha um importante papel na fase de reprodução peixes, e nos fazem refletir obre a necessidade de estudos mais aprofundados que auxiliem na explicação das respostas, e mostrem qual a ação deste composto na fisiologia reprodutiva, e fortalece a ideia de que a nutrição de reprodutores é uma diretriz que necessita de atenção a fim de que a reprodução e produção de formas jovens sejam realizadas com cada vez mais eficiência.