UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho" - UNESP
CENTRO DE AQUICULTURA DA UNESP (CAUNESP)
São Paulo State University - Aquaculture Center

TESE DE DOUTORADO

TÉCNICAS DE MANIPULAÇÃO CROMOSSÔMICA NO LAMBARI (*Astyanax altiparanae*)

Nivaldo Ferreira do Nascimento
Biólogo

Jaboticabal
São Paulo, Brasil
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Orientadora: Profa. Dra. Laura Satiko Okada Nakaghi
Coorientador: Dr. George Shigueki Yasui

Tese de Doutorado apresentada ao programa de Pós-graduação em Aquicultura, do Centro de Aquicultura da UNESP, campus de Jaboticabal, como parte das exigências para obtenção do título de Doutor em Biologia de Organismos Aquáticos e Aquicultura.

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“Ninguém pode ser sábio de estômago vazio”

George Eliot

“Aquele que ousa perder uma hora de seu tempo não sabe o valor da vida."

Charles Darwin

“Olho por olho, e o mundo acabará cego”

Mahatma Gandhi

“Se eu vi mais longe, foi por estar sobre ombros de gigantes. ”

Issac Newton

“A paz é a única forma de nos sentirmos realmente humanos. ”

Albert Einsten

“Façamos da interrupção um caminho novo. Da queda um passo de dança, do medo uma escada, do sonho uma ponte, da procura um encontro! ”

Fernando Sabino

“Quem estará nas trincheiras ao teu lado?

- E isso importa?

- Mais do que a própria guerra.”

Ernest Hemingway

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Resumo geral

O objetivo deste estudo foi aplicar técnicas de manipulação cromossômica para obtenção de indivíduos tetraploides e ginogenéticos de lambari (*Astyanax altiparanae*). Para tetraploidização, foram aplicados diferentes choques de temperatura (40°C por 2 minutos) com 16, 18, 20, 22, 24 e 26 minutos pós-fertilização (mpf) para inibição da clivagem. A análise em citometria de fluxo mostrou que o choque aplicado com 26 mpf garantiu a maior porcentagem de tetraploides (94,55%), sendo os resultados também confirmados por esfregaço sanguíneo e contagem dos cromossomos. Para indução de ginogenéticos foi primeiramente avaliada diferentes doses de irradiação UV (40, 80 e 120 mj/cm^2) para inativação dos espermatozoides, sendo verificado que a dose de 80 mj/cm^2 foi suficiente para assegurar a ativação dos oócitos e obtenção de larvas 100% haploides. Posteriormente, para restaurar a diploidia foi aplicado choque de temperatura (40°C por 2 minutos) para evitar a liberação do segundo corpúsculo polar. A análise molecular confirmou que os peixes ginogenéticos apresentam herança exclusivamente materna e apresentam número de cromossomos idêntico ao grupo controle. Em relação a razão sexual, a grande quantidade de fêmeas obtidas sugere que o sistema genético de determinação sexual seja homogamético para fêmeas (XX; XY). Este foi o primeiro protocolo de ginogênese em uma espécie da região Neotropical e primeiro de tetraploidização em uma espécie da família Characidae. Portanto, os resultados são inovadores e poderão ser aplicados em futuros estudos de manipulação cromossômica em espécies nativas.

Palavras-chave: Aquicultura, biotecnologia, poliploidia, tetraploidia, ginogênese.

General Abstract

The aim of this study was to apply techniques of chromosome set manipulation to obtain gynogenetic and tetraploid individuals in lambari (*Astyanax altiparanae*). For tetraploidization, it was applied different temperature shocks (40°C for 2 min) at 16, 18, 20, 22, 24 and 26 minutes post-fertilization (mpf) to inhibit the cleavage. The flow cytometric analysis showed that heat shock applied at 26 mpf enabled the highest percentage of tetraploids (94.55%), which were also confirmed by nuclear diameter of erythrocytes and chromosome counting. For gynogenetic induction, first it was evaluated the best UV-dosage for inactivation of sperm DNA (40, 80 and 120 mj/cm^2) and it was verified that the dosage of 80 mj/cm^2 ensure oocyte activation and 100% haploid larvae. Afterwards, to restore the diploidy it was applied a temperature shock (40° for 2 min) to avoid the extrusion of the 2nd polar body. The molecular biology confirmed that that gynogenetic fish present exclusive maternal inheritance e also showed the same number of chromosomes of the control group. Sex ratio showed increased amount of females, which suggest that the genetic system of sexual determination are homogametic for females (XX; XY). This is the first protocol of gynogenesis in a species of the Neotropical region and the first tetraploidizaiton in a species of the family Characidae. Therefore, the results are innovative and could be applied in future studies of chromosome set manipulation in native species.

Keywords: Aquaculture, biotecnhology, polyploidy, tetraploidy, gynogenesis.

1. Introdução geral

Nas últimas décadas, a aquicultura mundial vem apresentando um crescimento constante. No Brasil, este fato foi observado pelo recente relatório do IBGE (2016). Em 2015, o valor de produção foi de R$ 4,39 bilhões, com a maior parte oriunda da criação peixes (69,9%), seguida pela criação de camarões (20,6%). No que se refere aos peixes, a produção alcançou 483,24 mil toneladas, um aumento de 1,5% em relação ao ano anterior.

No entanto, com a queda da pesca extrativista e a crescente demanda por alimentos, a produção de organismos aquáticos provenientes da aquicultura deverá aumentar ainda mais. Assim, a aquicultura terá que dobrar sua produção nas próximas décadas, o que deverá ser feito utilizando menos espaço e água, principalmente devido às pressões referentes à urbanização, aumento populacional e mudanças climáticas. Neste aspecto, o emprego da Biotecnologia aplicada é um passo imprescindível para alcançar esses objetivos e suprir a crescente demanda (GUI, 2015). Neste cenário, a biotecnologia também contribui para uma aquicultura mais sustentável (GUI, 2015). Primeiramente, pode-se aumentar a produtividade e gerar produtos de melhor qualidade, diminuindo assim a necessidade por mais espaço e o consumo de água (FLETCHER et al., 2005; RATHER et al., 2011). Também é possível produzir organismos mais resistentes a doenças, permitindo um menor uso de medicamentos e produtos químicos que são nocivos à saúde humana e ao ambiente (ELASWAD e DUNHAM, 2017). Além disso, é possível a criação de peixes estéreis (NASCIMENTO et al., 2017), os quais, caso escapem para o meio ambiente, gerarão menos impacto ambiental (PIFERRER et al., 2009).

Estes são apenas alguns exemplos de como a biotecnologia pode ajudar no desenvolvimento da aquicultura de forma consistente e sustentável. Ao longo das últimas décadas, diversas tecnologias foram desenvolvidas e dentre estas, tem ganhado destaque a manipulação

cromossômica, principalmente a poliploidia através da produção de peixes triploides (ARAI, 2001).

2. Poliploidia e produção de peixes triploides

Os organismos aquáticos apresentam dois conjuntos de cromossomos em cada célula, um de origem materna (n) e outro de origem paterna (n) (LUTZ, 2003). Um organismo poliploide (poli=muitos; ploide=ploidia) apresenta um ou mais conjuntos de cromossomos além do considerado normal para espécie (PIFERRER et al., 2009). Assim, um organismo triploide, tetraploide e hexaploide são poliploides com três, quatro e seis conjuntos de cromossomos, respectivamente (DUNHAM, 2004). Animais poliploides podem ser encontrados na natureza (DUNHAM, 2004) e sua origem é devido ao processo de evolutivo (BORIN et al., 2002). No entanto, eles também podem ser artificialmente induzidos (ARAI, 2001).

Em peixes, a triploidizacão tem sido a técnica mais empregada, principalmente devido às grandes vantagens em relação aos organismos diploides (ARAI, 2001). Vale destacar a possibilidade de esterilização, maior crescimento somático, maior sobrevivência (devido à baixa agressividade) e melhor eficiência e conversão alimentar (LUTZ, 2003; FELIP et al., 2009). Além disso, pela geração de peixes estéreis, esta técnica diminui os riscos de contaminação genética ambiental quando espécies exóticas, híbridas ou transgênicas estão sendo produzidas (LUTZ, 2003).

A esterilidade dos triploides está relacionada a etapa reducional da meiose durante a gametogênese, pois apresentam conjunto ímpar de cromossomos (3n), o que, consequentemente, compromete esse processo (PIFERRER et al., 2009). Assim, caso sejam estéreis, a energia que

seria empregada para a produção dos gametas e comportamentos reprodutivos é direcionada para o crescimento somático (FELIP et al., 2009).

A técnica comumente empregada para induzir triploides é através da inibição da liberação do segundo corpúsculo polar, por meio de choques de temperatura, químico ou de pressão (ARAI, 2001). Essa técnica, embora eficaz, pode não ser 100% efetiva. Em *Astyanax altiparanae*, por exemplo, o choque de temperatura levou a produção de 92% de peixes triploides e 8% diploides (ADAMOV et al., 2017). Adicionalmente, os choques aplicados podem afetar negativamente a sobrevivência e o crescimento dos organismos triploides (MALISON et al., 1993; OPSTAD et al., 2013). Assim, como solução para esses problemas, pode-se simplesmente cruzar machos tetraploides com fêmeas diploides, evitando os efeitos deletérios dos choques supramencionados.

3. Produção de peixes tetraploides

A tetraploidização é uma técnica de manipulação cromossômica onde os indivíduos possuem quatro conjuntos de cromossomos (WEBER et al., 2015) e pode ser aplicada na produção em massa de peixes triploides (NAM e KIM, 2004). Para indução de peixes tetraploides são utilizadas estratégias similares à triploidização, como choques de temperatura, químico ou de pressão (ZHANG e ONOZATO, 2004; PIFERRER et al., 2009). No entanto, os choques são aplicados posteriormente, em diferentes fases do ciclo celular antes da primeira mitose, atuando na destruição dos filamentos do fuso mitótico e impedindo a divisão celular (ZHANG e ONOZATO, 2004).

Em comparação com a triploidização, protocolos para indução de peixes tetraploides são escassos (PIFERRER et al., 2009) e dentre estes, geralmente é observada baixa porcentagem e sobrevivência (SAKAO et al., 2006). Além disso, para que sejam empregados na produção em massa de peixes triploides, é necessário que os tetraploides sejam viáveis, ou seja, produzam

gametas diploides com qualidade e capacidade de fertilização (ZHAO et al., 2014). No entanto, tetraploides com gametas viáveis também são escassos, sendo limitados para algumas espécies (YOSHIKAWA et al., 2008). Em *Misgurnus mizolepis*, por exemplo, de 48 machos tetraploides, somente 3 (6%) produziram espermatozoides diploides, sendo o restante haploide (56%) ou aneuploide (15%). Posteriormente, os gametas diploides obtidos foram utilizados com sucesso para produção de progênie 100% triploide com altas taxas de fertilização (NAM e KIM, 2004).

Portanto, como observado acima, estudos com tetraploidização em peixes são menos comuns e devem ser estimulados. Vale destacar ainda, que além de serem utilizados para a produção de peixes triploides, gametas diploides provenientes de tetraploides são muito úteis para outros estudos de manipulação cromossômica, como: 1) produção de neo-tetraploides por meio da fertilização de um oócito haploide por um espermatozoide diploide e choque de temperatura para retenção do segundo corpúsculo polar (FUJIMOTO et al., 2010); 2) androgênese, pela inativação do material genético do oócito e posterior fertilização com espermatozoide diploide (YASUI et al., 2010) e 3) ginogênese, pela fertilização de oócitos diploides com sêmen irradiado (LI et al., 2013).

4. Produção de peixes ginogenéticos

Ginogênese é o termo que descreve a reprodução uniparental ou partenogenética (KOMEN e THORGAARD, 2007) com herança materna, ou seja, o genoma paterno é inativado (DUNHAM, 2004), empregando-se radiação ultravioleta (UV), radiação gama ou raios X. No entanto, a inativação por ultravioleta (UV) é mais comum, pois além de ser eficiente, apresenta baixo custo e simplicidade na operação. A ativação dos oócitoss com espermatozoides irradiados leva ao desenvolvimento de embriões haploides, os quais apresentam diversas malformações e morrem logo após a eclosão, condição conhecida como "síndrome haploide" (PAN et al., 2017). Portanto,

o estado diploide deve ser restaurado, o que pode ser realizado com choques de temperatura, químico ou de pressão (DUNHAM, 2004). Dependendo do modo de diploidizacão, a ginogênese pode ser meiótica (inibição da liberação do segundo corpúsculo polar) (ZOU et al., 2011) ou mitótica (bloqueio da clivagem) (MENG et al., 2016).

Organismos ginogenéticos são interessantes na aquicultura, pois podem ser empregados para a geração de populações monossexuais ou clonais (DUNHAM, 2004). Outra importante aplicação desta técnica é a elucidação do mecanismo genético de determinação sexual de uma espécie (KOMEN e THORGAARD, 2007). Embora o mecanismo de determinação sexual em peixes seja multifatorial, algumas espécies apresentam sistemas de determinação sexual similares ao de mamíferos e aves (presença de cromossomos sexuais simples), no qual: 1) o macho é heterogamético (XY – macho, XX – fêmea) e 2) aquele no qual a fêmea é heterogamética (ZW – fêmea, ZZ – macho) (WHITEHEAD et al., 2012). Assim, como ocorre duplicação do material genético, caso o sexo homogamético determine fêmeas, os ginogenéticos serão todos XX (fêmeas), como observado para *Gasterosteus aculeatus* (SAMONTE-PADILLA et al., 2011) e bacalhau do Atlântico (*Gadus morhua*) (WHITEHEAD et al., 2012). Do contrário, se as fêmeas forem o sexo heterogamético (ZW), ambos os sexos serão observados nas proles (ZZ e WW), como observado para Siberian sturgeon (*Acipenser baeri* Brandt) FOPP-BAYAT et al., (2010). Portanto, a ginogênese é uma interessante ferramenta para o desenvolvimento de estratégias para a produção de lotes monosexuais em espécies nativas, e dentre estas, se destaca o lambari (*Astyanax altiparanae*), alvo de diversos estudos na área de biotecnologia aplicada a aquicultura.

5. Espécie de estudo

O lambari (*Astyanax altiparanae*) é uma espécie nativa com grande importância para aquicultura, sendo apreciada para consumo humano ou como isca viva na pesca esportiva (PORTO-FORESTI et al., 2005). Esta espécie pertence à família Characidae, maior grupo de peixes no Brasil e ao gênero *Astyanax*, o qual compreende aproximadamente 100 espécies distribuídas na região Neotropical, sendo vulgarmente conhecida como lambari, tambiú piaba ou maturi (LIMA, 2003; MIRANDE, 2010). No *A. altiparanae* as fêmeas são maiores e os machos apresentam espículas na nadadeira anal. Devido ao pequeno porte (8 a 10 cm), facilidade de reprodução e rusticidade, o lambari vem se destacando como um organismo modelo para diversos estudos de biotecnologia (YASUI et al., 2015).

Assim, com o objetivo de padronizar protocolos de reprodução e técnicas biotecnológicas, esta espécie vem sendo alvo de diversos estudos nos últimos anos. Inicialmente, um protocolo de manipulação dos gametas e fertilização artificial foi estabelecido (YASUI, G. S. et al., 2015), o que possibilitou estudos detalhados da embriogênese, como as taxas de clivagem e verificação do momento exato da expulsão do segundo corpúsculo polar (PEREIRA-SANTOS et al., 2016). Tais resultados viabilizaram protocolos de manipulação cromossômica, como a indução (ADAMOV et al., 2016) e larvicultura de peixes triploides em sistema laboratorial (BERTOLINI et al., 2018). Em estudo recente, foi observado que peixes triploides desta espécie, em especial fêmeas, apresentam esterilidade (NASCIMENTO et al., 2017a), maior crescimento e excelente rendimento de carcaça (NASCIMENTO; et al., 2017b). Ou seja, a produção de proles 100% fêmeas triploides e estéreis seria um passo importante para incrementar a produção e diminuir os riscos de introgressão em caso de escapes na natureza, garantindo uma aquicultura mais rentável e sustentável.

Portanto, para alcançar esse objetivo, a técnica de ginogênese e tetraploidização podem ser utilizadas conjuntamente. Primeiramente, para o desenvolvimento de estudos de manipulação do sexo fenotípico, conhecimentos básicos da base genética de determinação sexual da espécie são necessários (DEVLIN e NAGAHAMA, 2002), o que é possível através da ginogênese (OTTERÅ et al., 2011; SHELTON e MIMS, 2012). No lambari, por exemplo, caso o sistema de determinação sexual seja heterogamético XY, a prole ginogenética será totalmente composta por fêmeas (XX). Este dado, aliado a um protocolo eficaz de tetraploidização, poderão ser aplicados para a produção em massa de peixes triploides e posteriormente para a geração de pseudo-machos tetraploides (i.e. machos XXXX). Estes, caso produzam espermatozoides XX, poderão reproduzir com fêmeas que também irão produzir oócitoss X. Assim, com um simples cruzamento, proles 100% triploides fêmeas (XXX) poderão ser obtidas.

6. Objetivos

Geral: Obter proles de lambari tetraploides e ginogenéticos, o que facilitará a produção de monosexos e peixes triploides no lambari (*Astyanax altiparanae*).

Específicos:

1) Obter peixes tetraploides através da inibição da clivagem empregando choques de temperatura;

2) Fertilizar oócitos com sêmen inativado com irradiação UV e reconstituir a ploidia por choque térmico, obtendo-se assim animais ginogenéticos;

3) Confirmar a paternidade dos ginogenéticos por biologia molecular;

4) Elucidar o mecanismo de determinação do sexo do lambari, através da análise da proporção sexual nos ginogenéticos.

7. Estruturação da tese

Tendo em vista os objetivos acima, esta tese foi dividida em dois capítulos. No **capítulo I** foi obtido um eficiente protocolo de tetraploidização utilizando choque de temperatura, sendo observada uma grande porcentagem de indivíduos tetraploides (94,55%). Os peixes foram mantidos até a vida adulta e os resultados confirmados por citometria de fluxo, esfregaço sanguíneo e citogenética.

No **capítulo II** é estabelecido um protocolo de indução a peixes ginogenéticos. Inicialmente, é estabelecida uma faixa de irradiação ideal para inativação dos espermatozoides que garanta a fertilização. Posteriormente, a diploidia é restabelecida por meio de choque de temperatura e os peixes são criados até a fase adulta, quando é realizada a análise de proporção sexual e confirmação dos resultados por biologia molecular.

8. Referências

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Capítulo I: High percentages of tetraploids in the yellowtail Tetra *Astyanax altiparanae* induced by heat-shock: the first case of tetraploids in Neotropical characins

Abstract

Tetraploid fish are important as source of diploid gametes for polyploid production and can be induced by inhibition of the mitotic cell division. Besides its potential, very few protocols regarding tetraploidization and subsequent application in aquaculture exists. In the yellowtail tetra *Astyanax altiparanae*, triploids presents sterility and increased carcass yield suggesting then the generation of tetraploid for large-scale production of such triploids. In this study, we compare the efficacy of heat shock treatments (40° for 2 min) at 16, 18, 20, 22, 24 and 26 minutes post-fertilization (mpf) on tetraploid induction of *A. altiparanae*. Ploidy status was confirmed by flow cytometry, karyotyping and nuclear diameter of erythrocytes. The results showed that heat shock decreased the hatching rate, especially at 22 (19.12 ± 9.59%), 24 (15.99 ± 9.12%), and 26 mpf (10.32 ± 6.23%) in comparison with control group (62.16 ± 7.69%). The flow cytometric analysis showed that the control group and the treatments heat shocked at 16 and 18 mpf just presented diploid individuals. However, from 20 mpf onwards, tetraploid individuals were observed and the highest percentage arose at 26 mpf (94.55%). Our results indicate that heat shock treatment at 26 mpf (40°C for 2 min) is optimum for tetraploid induction in the *A. altiparanae*. Our data are innovative and the first report of tetraploids in family Characidae, presenting application in basic and applied sciences.

Keywords: biotechnology, chromosome manipulation, heat-shock, polyploidy.

1. Introduction

Triploid fish in aquaculture has attracted considered attention (ARAI, 2001; BENFEY, 2016), as may reduce the deleterious effects of early sex maturation due to functional sterility (TARANGER et al., 2010; HULATA, 2001). In such cases, the energy normally used for gonadal development are deviate into somatic tissue in triploids leading to increased growth and carcass yield (NASCIMENTO et al., 2017a; GOLPOUR et al., 2016; MORÓN-ALCAIN et al., 2017). Triploidization also reduces the environmental impacts caused by escaping of farmed fish, since breeding and introgression are avoided (BENFEY, 2015). Triploids are conventionally induced by temperature or pressure shocks few minutes after fertilization in order to suppress the extrusion of the 2nd polar body during meiosis (PIFERRER et al., 2009). However, induction of triploid fish in commercial hatcheries are difficult due to intensive labor, lower survival and the rise of abnormalities when shocks are applied (GARCIA et al., 2017b; STEFANO et al., 2007). In addition, most protocols of induced triploidization are not 100% effective (ADAMOV et al., 2017; GARCIA et al., 2017b), what does not permit the application in large-scale.

In this scenario, an interesting alternative for massive production of triploid fish is to use gametes source from tetraploid fish (e.g. when normal eggs are fertilized with diploid sperm from a tetraploid male)(NAM and KIM, 2004; WEBER et al., 2014; WEBER et al., 2015). Such a procedure avoid the deleterious effects of 2nd polar body retention giving rise to increased survival (CHOURROUT et al., 1986; LEBEDA and FLAJSHANS, 2015). Tetraploid fish can be artificially induced by suppressing the cleavage of the zygote also using thermal or pressure shocks (ZHANG and ONOZATO, 2004; PIFERRER et al., 2009). Furthermore, besides being used to produce triploid fish, diploid gametes from tetraploid lines can be applied in several other studies of chromosome set manipulation, such as neo-tetraploid induction (FUJIMOTO et al., 2010),

gynogenesis (LI et al., 2013) and androgenesis (YASUI et al., 2010; HOU et al., 2013), what emphasize the application in the field of basic sciences and conservation. However, tetraploidization is difficult to achieve (YOSHIKAWA et al., 2008) and viable lines are limited to a few species (PIFERRER et al., 2009).

In the Neotropical region, for example, tetraploid induction are reported only in a catfish species *Rhamdia quelen*, in which very low percentages of tetraploids arose and no tetraploid lines were established (GARCIA et al., 2017a). In this aspect, the yellowtail tetra *Astyanax altiparanae* is an interesting candidate to establish such a protocol, since this species has been used as model organism for basic and applied studies (PEREIRA-SANTOS et al., 2016; NASCIMENTO et al., 2017b; PIVA et al., 2018; SIQUEIRA-SILVA et al., 2015). This species is included in the family Characidae, which presents ~ 1100 species (ESCHMEYER and FONG, 2017) and is one of the most aquaculture species in South America, what emphasize the data obtained from the yellowtail tetra may have application for other related species. In this species, our group has established protocols for production and rearing of triploid fish. Firstly, to control the timing for fertilization, a protocol for in vitro fertilization was accomplished (YASUI et al., 2015) which enable studies on early embryology and chromosome set manipulation, like the extrusion of the second polar body (PEREIRA-SANTOS et al., 2016) triploid induction (ADAMOV et al., 2017) and first feeding within triploids (BERTOLINI et al., 2018). Afterwards, we observed that triploid fish present increased carcass yield (NASCIMENTO et al., 2017a) and the females are sterile (NASCIMENTO et al., 2017b), suggesting that mass production of triploid offspring of *A. altiparanae* are interesting for aquaculture purposes. However, besides the efficacy of our previous protocol using heat-shocking, we still not obtained massive offspring (100%) of triploid individuals (ADAMOV et al., 2017) and an effective method to achieve 100% of triploids is to use

gametes from a tetraploid individual. However, as far as we know, there is not a protocol to induce tetraploid individuals in this species or even in other Neotropical characins. Therefore, the aim of the study is to induce tetraploid fish in *A. altiparanae* using temperature shock.

2. Material and Methods

2.1. Ethics

This study was conducted according with the Guide for the Care and Use of Laboratory Animals at the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental (CEUA / CEPTA#0231.000070/2018-63).

2.2. Broodstock induction

The fishes (adult specimens of *Astyanax altiparanae*) used in this experiment were previously maintained in 1000m^2 earthen ponds at the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental/Instituto Chico Mendes de Conservação da Biodiversidade (CEPTA/ ICMBio), Pirassununga City, São Paulo State, Brazil (21°55'58"S, 47°22'31"W). The fish were fed twice a day with commercial diet (4200 kcal kg^{-1} and 45% crude protein). Procedures for artificial fertilization were performed according to YASUI et al., (2015) and NASCIMENTO et al., (2017a). Adult males (SL ~ 6 cm; n=3) and females (SL ~ 8 cm; n=3) were selected, anesthetized in eugenol solution (100 mg L^{-1}) and hormonally induced with a single injection of pituitary gland extract from common carp fish (3 mg kg^{-1} body weight). Approximately eight hours afterwards, when spawning behavior was observed (males following the females), the fish were then separated for gamete collection. Males were anesthetized as describe previously and sperm collected using a micropipette (Eppendorf, Hamburg, Germany) and immediately transferred to 1.5 mL tube containing 400 µL of modified Ringer's solution (NaCl 128.3 mM, KCl 23.6 mM, CaCl2 3.6 mM, $MgCl_2$ 2.1 mM). Sperm quality were evaluated

according to YASUI et al., (2015) and just samples with progressive motility higher than 80% were used. Females were also anesthetized and mature oocytes were stripped in to a Petri dish (90 mm diameter) covered with polyvinylidene chloride film (saran wrap, Alpfilm, São Paulo, Brazil).

2.3. Tetraploidization

Oocytes (~ 8000) from each female (triplicate) were inseminated using 90µL of sperm from different males, and the gametes were activated by adding 5 mL of water. The fertilized eggs (~ 8000) were then divided in seven plastic containers in which the bottom contained a 100-µm nylon mesh and was maintained in water with controlled temperature at 22 °C. One container was kept intact (diploid control group), and the others were subject to heat shock (40 °C; 2 min) at 16, 18, 20, 22, 24, and 26 min post-fertilization (mpf) (~1100 eggs in each treatment). After heat-shocking, all containers were transferred to water with constant aeration and controlled temperature at 26°C. Such procedures were performed in triplicates.

2.4. Development and fish rearing

A small aliquot of about 150 eggs from every experimental group (plastic container) was placed in a 90-mm Petri dish for observation of survival at 2-cell, blastula, gastrula, and somite stages, as well hatching and subsequent normal and abnormal larvae using a stereomicroscope (Nikon SMZ 1500,Tokyo, Japan). Digital images were obtained using a CCD camera (Nikon Ds-Fi, Tokyo, Japan) with the Nis-Ar Elements software (Nikon, Tokyo, Japan). At hatching, larvae from each Petri dish were randomly selected for ploidy analysis by flow cytometry (n = 60 for each treatment).

The best treatment obtained were used to produce adult tetraploids. The larvae were reared in 40-L aquariums with temperature set at 28 °C and fed exclusively with *Artemia franciscana* nauplii twice a day until 10 days post-hatching (dph). After this period, the fish were fed with

powdered commercial food (4200 kcal kg^{-1} and 45% crude protein). Tanks were constantly monitored for cleaning and removal of dead fish. At 30 dph, the fish start to feed commercial pellets (1 mm, 91.62% dry matter, 45% crude protein, 8% crude fat, 2.8% crude fiber and mineral matter 12.10%) twice daily until apparent satiation. At four-months age, all remaining fish were analyzed by flow cytometry (using a small piece of the anal fin) and diploid and tetraploid individuals were identified and maintained separated in 40 L aquariums as described previously.

2.5. Flow cytometry

Samples used for flow cytometry were analyzed according to the protocol developed by XAVIER et al., (2017). The samples were lysed in a detergent solution (9.53 mM $MgSO_4.7H_2O$, 47.67mM KCl, 15 mM Tris, 74 mM Sucrose and 0.8% of Triton X-100) filtered through 30 μm nylon mesh and the extracted nuclei were stained with the 4',6 diamidino-2-phenylindole dihydrochloride (DAPI). The DNA content was then measured with a flow cytometer (CyFlow Ploidy, Analyzer, Partec, GMBh, Germany). The ploidy of all samples was confirmed by comparison with haploid control (spermatozoa from diploid males).

2.6. Blood analysis and chromosome preparations

To confirm the flow cytometric analysis, 5 diploid and 5 tetraploid individuals were randomly collected from the aquariums. The fish were anesthetized in eugenol solution (1 $g.L^{-1}$) and had the blood collected from caudal puncture using a syringe containing 1 drop of EDTA (5%). Afterwards, the blood smears were prepared by placing a drop of blood on a glass slide and dragging it from one side to another. The smears were stained with the rapid panotic kit (Laborclin, Pinhais, Brazil) and observed with an optic microscope (Nikon NI, Tokyo, Japan) at magnification of 400x. We use the software Nis-Ar Elements (Nikon, Tokyo, Japan) to take the microphotographs and the open software Image J (National Institutes of Health, USA,

http://rsb.info.nih.gov/ij/) for posterior analysis. Area (μm^2), perimeter (μm), major axis (μm) and minor axis (μm) were measured from nuclei of each image and compared the 2n and 4n fish. Afterwards, 3 diploid and 3 tetraploid fish were euthanized in eugenol solution (100 mg L^{-1}) for chromosome preparation, which were obtained according to (GOLD et al., 1990), followed by Giemsa staining.

2.7. Statistics

Results are shown as mean ± standard error. The data were checked for normality using the Liliefors test and analyzed by ANOVA. Data expressed as percentages were transformed in order to fit the assumptions of statistical variance homogeneity using the Levene test (BROWN & FORSYTHE, 1974). Analysis was performed using the software STATISTICA v. 10.0 (Statsoft, Tulsa, U.S.A.) and significance was set at $P < 0.05$.

3. Results

3.1. Early development

Data on development of heat-shocked embryos are detailed on Table 1. No differences between treatments were observed for survival rates at 2-cell (*P = 0.9952*), blastula (*P = 0.9998*) and gastrula (*P = 0.1340*) stages. However, at somite stage, the control group presented higher survival (70.20 ± 7.70%; *P = 0.0008*) and the 24 and 26 mpf treatments showed lower survival rate, with 24.56 ± 8.51% and 13.51 ± 7.05%, respectively. Similar results were observed for hatching rate (*P = 0.0007*), with higher values observed for control group (62.16 ± 7.69%) and the lower for the 22 (19.12 ± 9.59%), 24 (15.99 ± 9.12%), and 26 mpf (10.32 ± 6.23%) treatments, respectively. After hatching, the percentage of abnormal larvae was significantly higher (*P = 0.0009*) in embryos heated shocked at 22 (82.17 ± 6.66%), 24 (78.31 ± 7.28) and 26 mpf (79.01 ± 7.85%) than the control group (12.87 ± 4.46%).

3.2. Flow cytometry

The flow cytometric analysis showed that the control group and the treatments heat shocked at 16 and 18 mpf presented only diploid individuals (Table 1). However, in heat shock treatments from 20 to 26 mpf, tetraploid individuals were observed with the highest percentage with 26 mpf (94.55%) (Table 1).

1.1.3.3. Blood smear and chromosome counting

In comparison with diploid fish, erythrocyte nuclei from tetraploid fish presented significantly increased area (*P = 0.0018*), perimeter (*P = 0.0003*), and major axis (*P = 0.0002*); however, significantly lower circularity (*P = 0.0003*) were observed (Figure 1 A,B; Table 2). For minor axis, no differences were verified (*P = 0.6144*). Additionally, while diploids presented 50 metaphase chromosomes, tetraploid individuals had 100 chromosomes (Figure 1 C,D).

Table 1: Development of yellowtail tetra, *Astyanax altiparanae*, after heat shock treatment for induction of tetraploid fish.

Tratament (mpf)	2-cell (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatching (%)	Normal (%)	Abnormal (%)	Ploidy		
								2n	4n	4n (%)
Control	97.45 ± 1.41	92.88 ± 3.63	75.05 ± 6.31	70.20 ± 7.70^{a}	62.16 ± 7.69^{a}	87.13 ± 4.46^{a}	12.87 ± 4.46^{a}	60	0	0.00%
16	94.71 ± 3.95	90.58 ± 5.00	63.46 ± 9.69	46.88 ± 8.18^{ab}	37.43 ± 7.42^{ab}	50.41 ± 15.51^{ab}	49.59 ± 15.51^{ab}	58	0	0.00%
18	95.27 ± 3.34	90.90 ± 5.03	65.30 ± 8.46	47.01 ± 5.48^{ab}	34.07 ± 1.38^{ab}	62.91 ± 10.90^{ab}	37.09 ± 10.90^{ab}	57	0	0.00%
20	98.38 ± 0.33	92.20 ± 6.29	70.49 ± 7.24	48.43 ± 9.41^{ab}	34.10 ± 3.80^{ab}	37.87 ± 16.28^{ab}	62.13 ± 16.28^{ab}	41	16	28.07%
22	94.35 ± 3.54	88.46 ± 6.36	56.32 ± 11.95	35.45 ± 8.79^{ab}	19.12 ± 9.59^{b}	17.83 ± 6.66^{b}	82.17 ± 6.66^{b}	29	23	44.23%
24	94.34 ± 4.35	89.00 ± 6.07	49.23 ± 14.02	24.56 ± 8.51^{b}	15.99 ± 9.12^{b}	21.69 ± 7.28^{b}	78.31 ± 7.28^{b}	17	23	57.50%
26	92.9 ± 5.76	87.57 ± 7.14	34.30 ± 11.77	13.51 ± 7.05^{b}	10.32 ± 6.23^{b}	20.99 ± 7.85^{b}	79.01 ± 7.85^{b}	3	52	94.55%
P-value	*0.9952*	*0.9998*	*0.1340*	*0.0008*	*0.0007*	*0.0009*	*0.0009*		-	

Fertilized eggs were heat shocked at 16, 18, 20, 22, 24 and 26 mpf. After treatment, the eggs were incubated at 26°C until hatching. Distinct superscript letters indicates statistical differences by the Tukey multiple range test (ANOVA; $P < 0.05$). mpf: minutes post-fertilization.

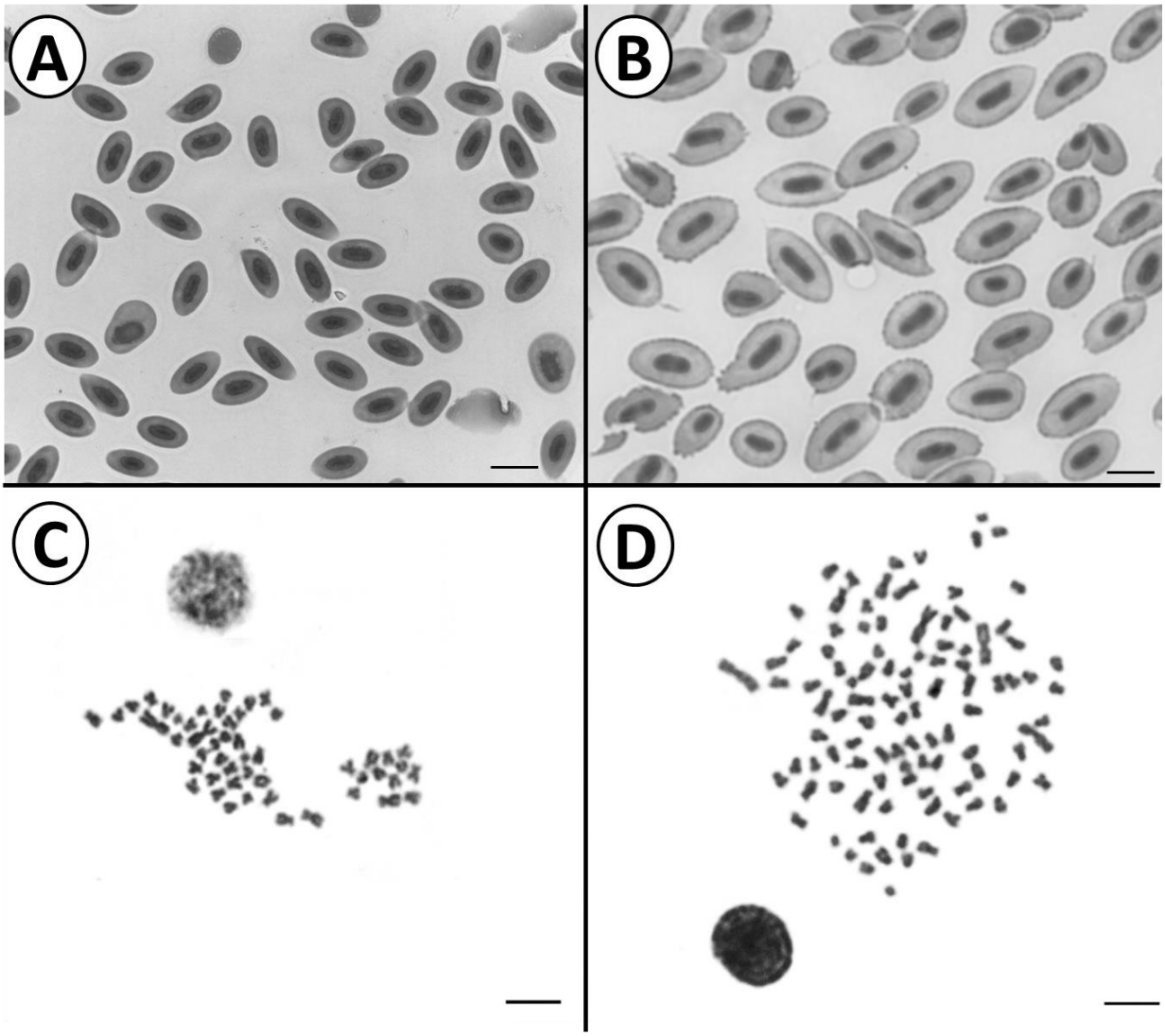

Figure 1: Blood smears and metaphase chromosomes of diploid (left) and tetraploid (right) individuals of yellowtail tetra *Astyanax altiparanae*. Erythrocytes nuclei from diploids (A) presented decreased size in comparison with tetraploids (B). The chromosome numbers are 50 (C) and 100 (D) in diploid and tetraploid, respectively. Scale: 10 µm.

Table 2: Nuclear measurements of erythrocytes from diploid and tetraploid *Astyanax altiparanae*.

Parameters	2n			4n			*P-value*
Area (μm^2)	16.15	±	0.89^a	23.34	±	0.82^b	*0.0018*
Perimeter (μm)	15.46	±	0.39^a	19.58	±	0.49^b	*0.0003*
Major axis (μm)	5.40	±	0.13^a	7.63	±	0.19^b	*0.0000*
Minor axis (μm)	3.78	±	0.12^a	3.91	±	0.05^a	*0.6144*
Circularity*	0.84	±	0.01^a	0.79	±	0.01^b	*0.0002*

Distinct letter in the same line indicate significant difference (ANOVA, $P < 0.05$).
Circularity: 4π * (area) / (perimeter)^2

4. Discussion

In the present study, high percentages of tetraploids were successfully obtained in *Astyanax altiparanae* by heat shocking. Those results are very interesting for aquaculture, since tetraploids can be used as a source of diploid gametes for mass production of sterile triploids without the negative effects of the shock treatment (GARCIA et al., 2017b; LEBEDA and FLAJSHANS, 2015). In *A. altiparanae,* previous studies showed that triploid females are sterile (NASCIMENTO et al., 2017b) presenting increased carcass yield (NASCIMENTO et al., 2017a), which supports mass production of triploid fish using tetraploid individuals.

Our previous study of early development in *A. altiparaane* showed that the first cleavage takes place at 43 min post-fertilization (at 22°C) and pronuclei fusion occurs within 10 mpf (PEREIRA-SANTOS et al., 2016). Therefore, the timing for heat shock were standardized based on a relative unit of embryological age (t0 or tau zero)(GOMELSKY, 2003) nearly at pro-metaphase of the first cleavage, reported as the optimum time for tetraploid induction in previous studies (SAKAO et al., 2006; FUJIMOTO et al., 2013). Additionally, we used a heat shock similar to the used for triploidization in *A. altiparanae* (40°C for 2 min), which indicated that such a temperature is considered sublethal during the early stages and resulted in increased triploid production (ADAMOV et al., 2017). The combination of those information gave support to our procedure which almost achieved 100% of tetraploids.

The decreased survival observed during tetraploidization are commonly reported in other species (SAKAO et al., 2006) and after hatching, several studies showed no survival at first feeding (HANIFFA et al., 2004; SAKAO et al., 2006; GIL et al., 2016). Lower survival of tetraploids may be related with mosaicism, aneuploidy, asynchronous development or high homozygosity (ZHANG and ONOZATO, 2004; FUJIMOTO et al., 2013; PANDIAN and

KOTEESWARAN, 1998). In our study, no mosaicism or aneuploidy were observed and the decreased survival can be addressed to factor other than chromosomal alterations, which need to be investigated in the future.

Studies also shows that the optimal protocol for tetraploid achievement depends also on egg batch (HERSHBERGER and HOSTUTTLER, 2007) or the species (PIFERRER et al., 2009). Salmonids, for example, appear to have low tolerance to ploidy alterations (diploidy to tetraploidy) and survival depends not only on the side effects of the tetraploid induction (SAKAO et al., 2006). On the other hand, second generation of tetraploid offspring seems to increase the survival and growth, like observed in Rainbow trout (*Oncorhynchus mykiss*)(CHOURROUT et al., 1986). Therefore, the achievement of adult tetraploids in *A. altiparanae* is very important and to confirm its potentiality, future evaluation of gametes, such as ploidy status and fertilization ability (ZHAO et al., 2014) is required. In this aspect, the production of diploid gametes could be significant for several other applications on chromosome manipulation, such as second generation of neo-tetraploids (FUJIMOTO et al., 2010), gynogenesis (LI et al., 2013) and androgenesis (YASUI et al., 2010; HOU et al., 2013).

Regarding the evaluation of tetraploid induction, the confirmation tools for ploidy evaluation are essential, since most protocols for ploypoid induction are not 100% effective. Several authors has successful used the erythrocytes nuclei measurements to confirm polyploidy in fishes (FLAJŠHANS et al., 2011; BORON, 1994), since such a method is considered simple and inexpensive. Here we show that erythrocytes nuclei measurement is an effective tool to confirm tetraploidy in *A. altiparanae*. Specifically, some authors stated that the axes of erythrocytes nucleus are generally considered the best prediction of ploidy in fish (KENANOĞLU et al., 2012). FLAJŠHANS et al. (2011), on the other hand, showed that area and perimeter were

the most accurate ploidy predictors. In our study, except for the minor axis, all other parameters were significantly different among diploid and tetraploid fish *A. altiparanae*.

Tetraploid fish was also identified by flow cytometry (a well-recognized, effective and accurate technique for nuclear DNA content analysis) (XAVIER et al., 2017; ALLEN, 1983) and chromosome counting (ALLEN JR et al., 1982). Therefore, the combined methods confirmed the efficacy of our protocol. Additionally, in the case that flow cytometry is not available, the blood smear or the cytogenetic method could be an interesting alternative. In conclusion, we established a protocol to produce highest percentages of tetraploid within Neotropical species, using the yellowtail tetra *A. altiparanae* as a model fish. The tetraploids were optimized using temperature shock at 26 mpf (40°C for 2 min) followed by incubation at 26°C. Such a protocol is innovative for the species and highly applicable for basic and applied sciences for the species and other related species.

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Capítulo II: The first case of induced gynogenesis in Neotropical fishes using the yellowtail tetra (*Astyanax altiparanae*) as a model organism

Abstract

Gynogenesis is a chromosome manipulation technique applicable on the production of all-female populations, generation of inbred or clonal lines and identification of genetic sex determination in fish. Therefore, the aim of this study was induce meiotic gynogenesis in *Astyanax altiparanae* and determine the genetic basis of sex determination. Firstly, we evaluated the best UV-irradiation dosage in order to inactivate sperm genome, preserving the capacity of egg activation without the genetic material contribution to the offspring. After the fertilization using the inactivated sperm, diplodization was achieved by heat-shock (40°C; 2 min) by suppression of the 2nd polar body extrusion. Diploid control and gynogenetic individuals were raised until 240 days. The gynogenetic offspring were confirmed by genotyping using microsatellite markers and the sex ratio of gynogenetic progenies suggests that female presents homogametic sex determination mechanism (XX; XY). This was the first protocol of induced gynogenesis in a Neotropical fish and such a procedure may be used to produce clonal linage of fish and for future mass production of monosex populations in *A. altiparanae* and other related species.

Keywords: chromosome set manipulation, sex determination, sex ratios, microsatellite marker.

1. Introduction

Gynogenesis is a term used for the generation of uniparental or partenogenetic offspring derived exclusively from the maternal genome (KOMEN and THORGAARD, 2007). In fish, this reproductive biotechnology may be artificially induced and involves two steps including 1) the inactivation of sperm genome, and 2) diploidization in order to produce viable offspring. Inactivation of sperm genome is generally achieved using ultraviolet (UV) irradiation causing structural damage and leading to no functional contribution of the paternal genome in the resultant offspring (LEBEDA et al., 2014). However, the inactivated spermatozoa may still present motility and the ability to activate embryo development. Diploidization is then necessary in order to produce viable gynogenetic progenies, since haploid status in fish results in mortality during the early stages (TVEDT et al., 2006; PAN et al., 2017). Diploidization is then achieved by thermal or pressure shocks (DUNHAM, 2004) in order to suppress of the meiosis II (ZOU et al., 2011) or mitosis I (MENG et al., 2016), although the first procedure results in increased survival during early and juvenile stages.

Gynogenetic fish are interesting for aquaculture to produce monosex populations and clonal lineages (KOMEN and THORGAARD, 2007). This reproductive biotechnique can also be applied to elucidate the mechanism of sex-determining in fish (DEVLIN and NAGAHAMA, 2002). In this regard, although sex determination in fish is multifactorial, it is known that some fish species present predominantly the same genetic mechanism observed for birds or mammals, where 1) the male are heterogametic and classified as XY and females are XX or 2) the female are heterogametic and classified as ZW and males are WW (WHITEHEAD et al., 2012). Consequently, if the homogametic sex determines females, all gynogenetic progenies will be females (XX), as observed for *Gasterosteus aculeatus* (SAMONTE-PADILLA et al., 2011) and

Gadus morhua (WHITEHEAD et al., 2012). On the other hand, if the females are determined by the heterogametic sex (ZW), both sexes will be observed in the offspring (ZZ and WW), as verified for Siberian sturgeon *Acipenser baeri* Brandt (FOPP-BAYAT, 2010).

Such an information may be interesting in other fish species such as the yellowtail tetra (*Astyanax altiparanae*) a Neotropical species with importance to aquaculture. Additionally, due to its small size (8-10 cm), facility of reproduction, rearing and ability to adjust into ponds and aquariums, such species is also being used in basic and applied sciences as a model organism. Several studies focusing on the reproductive biotechniques of *A. altiparanae* have being developed in the recent years. Firstly, a protocol for in vitro fertilization was established (YASUI et al., 2015), giving rise to a detailed study of the early development, and the moment of the 2nd polar body extrusion (PEREIRA-SANTOS et al., 2016). Such results were essential to generate protocols for triploidization (ADAMOV et al., 2017) and larviculture of triploid fish (BERTOLINI et al., 2018). Recently, it was observed that triploid females present increased carcass yield (NASCIMENTO et al., 2017a) and sterility (NASCIMENTO et al., 2017b), although males are smaller and are not sterile. Such a data suggests that monosex female population is more attractive in this species. Moreover, aquaculture as a finfish with efficient yield production require the generation of 100% triploid females offspring. In order to establish an effective strategy to produce monosex female populations, firstly is necessary to understand the mechanism of sex determination, which can be obtained by gynogenesis (KOMEN and THORGAARD, 2007) as mentioned above. In the past decades, the gynogenese technology has been applied for several fishes species (KOMEN and THORGAARD, 2007), however, there is not a protocol established for Neotropical species. Hence, the aim of this study was to establish a protocol for induce a meiotic gynogenesis in the yellowtail tetra *A. altiparanae*.

2. Materials and methods

2.1. Ethics

All the procedures were performed according to the Guide for the Care and Use of Laboratory Animals at the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental (CEUA / CEPTA#0231.000070/2018-63).

2.2. Animals and reproduction

Adult specimens of *Astyanax altiparanae* (SL ~ 6 cm for males; SL ~ 8 cm for females) used in the experiments were previously maintained in 1000 m^2 earthen ponds at the "Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental/Instituto Chico Mendes de Conservação da Biodiversidade (CEPTA/ICMBio)" in Pirassununga, Brazil (21°55'58"S, 47°22'31"W). The fish were fed twice a day with commercial diet (4200 kcal kg^{-1} and 45% crude protein).

Sexually mature couples of *A. altiparanae* (n=24) were selected for artificial reproduction according to the criteria of YASUI et al. (2015). Males were selected by the presence of bony hooks in the anal fins. Females were selected based on morphological appearance, presenting a reddish papilla and a soft, protuberant ventral region. Both males and females were hormonally induced for spawning with a single injection of carp pituitary extract at 3 mg kg^{-1}. Ten hours afterwards, the fish were anesthetized in menthol solution at 100 mg L^{-1} (Êxodo Científica, Hortolândia, Brazil) and the semen was collected in 1000-µL pipette (Eppendorf, Hamburg, Germany) and immediately transferred to 400 µL of modified Ringer's solution, (128.3 mM NaCl,

23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$) in order to immobilize the sperm (YASUI et al., 2015). Sperm motility were evaluated according to our previous protocols (YASUI et al., 2015; PEREIRA-SANTOS et al., 2016; PEREIRA-SANTOS et al., 2017) and samples with progressive motility higher than 80% were maintained at 2.5°C for subsequent fertilization trials. The oocytes were stripped in a 90 mm-Petri dish covered with polyvinylidene chloride film (saran wrap, Alpfilm, São Paulo, Brazil), and immediately used for fertilization trials.

2.3. Optimization of UV irradiation to genetic inactivation of sperm nucleus

For genetic inactivation of the sperm genome, an aliquot of 500 µL of the sperm were homogeneously distributed on a glass Petri dish with 90 mm diameter and subjected to UV irradiation in a irradiation chamber (see FUJIMOTO et al., 2007). The sperm were irradiated at 0 (control), 40, 80 and 120 mJ/cm^{-2}, respectively, which intensity was confirmed using a UV-meter (254 nm) (VS-2, SAKURA SEIKI Co. Tokyo, Japan). All the procedures were performed in a chamber in dark conditions in order to avoid genetic photoreactivation.

Sub aliquots of 30 µl of irradiated sperm were removed in each moment (0, 40, 80 and 120 mJ/cm^{-2}) and used for in in vitro fertilization. Aliquots of ~150 oocytes (20 µL of oocytes) were collected using a cut tip, and placed on a 90-mm Petri dish containing the plastic film (see above). The oocytes were inseminated and the gametes were activated by addition of 5 mL of dechlorinated tap water. The eggs were incubated in Petri dishes at room temperature (26 °C) and partial water changes were performed during incubation (until hatching). The development was observed on a stereomicroscope (Nikon SMZ 1500, Tokyo, Japan) coupled with the CCD camera (Nikon DS-F1, Nikon, Toktyo, Japan) and the software Nis-Ar-Elements (Nikon, Tokyo, Japan). Percentages

of the main developmental stages, including unfertilized eggs, 2-cell, blastula, gastrula, and somite stage, as well hatching of normal and abnormal larvae were measured (see PEREIRA-SANTOS et al., 2016). Six couples of *A. altiparanae* were used to perform this assay (six replications).

The quality of irradiated sperm was also evaluated. For motility analysis, 1 µL of the sperm from each treatment of irradiation was pipetted on a Makler chamber (Selfi-Medical Instruments, Haifa, Israel) previously coated with 0.1% bovine serum albumin. Activation was achieved with 30-fild dilution with distillated water. Videos sequences were captured with a microscope (Nikon-Eclipse Ni, Tokyo, Japan) equipped with a CCD camera (Nikon DSRi2, Nikon, Tokyo, Japan) with the NIS-Ar Elements software (Nikon, Tokyo, Japan). Motility analysis was performed using the Computer-Assisted Sperm Analysis (CASA) in the ImageJ software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/)(WILSON-LEEDY and INGERMANN, 2007) after standardization for the species (unpublished data). The parameters of motility (%), VCL (curvilinear velocity), VAP (average path velocity) and VSL (straight line velocity) were analyzed. In this experiment, four males were used (four repetitions).

Sperm viability was evaluated by flow cytometry, using the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit; Molecular probes, Eugene, OR, USA). Samples of 150 µL at concentration of 3.6×10^6 spermatozoa mL^{-1} were stained with SYBR-14 and PI according to manufacturer instructions and incubated for 10 min. Afterwards, 10,000 events from each samples were analyzed by flow cytometry (C6 Accuri; BD Biosciences, San Jose, CA, USA). SYBR-14 was detected using the FL1 filter (530 ± 15 nm) and PI using FL3 filter (> 670 nm). Gating of the forward-scatter (FSC) versus side-scatter (SSC) plots were performed to exclude non-sperm events (debris). Subsequently, gated events were views on a scatter plot (FL1 vs FL3) and membrane integrity assessed based on the percentage sperm stained with SYBR-14

from those stained with SYBR-14 and IP. The optimum sperm irradiation dosage obtained here was used in the next experiments.

2.4. Heat Shock to induce diploid meiotic gynogenesis and fish rearing

The procedures of heat shocking to inhibit the extrusion of the second polar body were performed according to Adamov and collaborators (2017) (shock at 2 min post-fertilization (mpf) at 40°C during 2 min). Other five sexually mature couples of *A. altiparanae* were selected and induced to reproduction as described previously (Five repetitions). Gametes were extruded and groups of oocytes (~8,000) from each female were divided into four aliquots and treated as follows: 1) fertilized with non-irradiated sperm (diploid control); 2) fertilized with non-irradiated sperm and heat shocked (triploid control); 3) fertilized with irradiated sperm with 80 mJ/cm^{-2} (haploid group) and 4) fertilized with irradiated sperm (80 mJ/cm^{-2}) and heat shocked (presumptive ginogenetic diploid).

Afterwards, a small aliquot of ~150 eggs from each of five experimental group was placed in a 90-mm Petri dish at room temperature (26 °C) for observation of developmental stages (fertilization, 2-cell, blastula, gastrula, somite stage and hatching, with normal and abnormal larvae) (see PEREIRA-SANTOS et al., 2016), which were evaluated with at the stereomicroscope (Nikon SMZ 1500, Tokyo, Japan) coupled with the CCD camera (Nikon DS-F1, Nikon, Tokyo, Japan) and the software Nis-Ar-Elements (Nikon, Tokyo, Japan). Partial water changes from Petri dishes were performed until hatching, when samples were collected for flow cytometric analysis.

Eggs from diploid control and gynogenic diploid treatments were also incubated and reared in 40-L aquariums with constant aeration and controlled water temperature at 26°C (Four

repetitions, R1, R2, R3 and R4). The larvae were fed to satiation exclusively with *Artemia franciscana* nauplii twice a day until 10 days post-hatching (dph) and afterwards, additional powdered commercial food containing 4200 kcal kg^{-1} and 45% crude protein was included. Tanks were constantly monitored for cleaning and removal of dead fish. At 30 dph, the fish started to feed the commercial pellets of 1 mm diameter, constituted by 91.62% of dry matter, 45% crude protein, 8% crude fat, 2.8% crude fiber and mineral matter 12.10%, twice daily to apparent satiation until 240 days. These fish were used for genotyping and sex ratios analysis.

2.5. Ploidy Confirmation

Samples of hatched larvae (n=20 from each repetition) obtained in section 2.3 and 2.4 were evaluated for ploidy status by flow cytometry according to the protocol developed by Xavier et al., (2017). Firstly, enucleation was achieved using a lysing solution (9.53 mM MgSO4.7H2O, 47.67mM KCl, 15 mM Tris, 74 mM Sucrose and 0.8% of Triton X-100). DNA staining was then achieved using the 4',6 diamidino-2-phenylindole dihydrochloride (DAPI) in D-PBS - Dulbecco's Phosphate Buffered Saline (9.6g/L) and then filtered through 30 μm nylon mesh (Celltrics, Partec, GMBh, Germany). The DNA content was then measured with a flow cytometer (CyFlow Ploidy, Analyzer, Partec, GMBh, Germany). The ploidy of all samples treatments was confirmed by comparison with haploid control of spermatozoa from diploid males of yellow tetra.

2.6. Genotyping

Twenty-seven presumptive diploid gynogenetic individuals were randomly sampled from repetitions R2 (n = 5), R3 (n = 11) and R4 (n=11) obtained in section 2.4. Genomic DNA was isolated from the skeletal muscle by using the kit "E.Z.N.A. Tissue DNA kit® (Omega)" according to manufacturer instructions. Polymerase chain reaction (PCR) amplification was performed in a total volume of 30 µL using 200 ng of genomic DNA, recombinant Taq (Invitrogen) and the specific primers for microsatellite regions of yellow tetra designed by (ZAGANINI et al., 2012). The amplification reactions were performed using 35 cycles of 45 s at 94°C, 30 s at 55°C and 45s at 72°C. Amplicons were separated by 12% polyacrylamide gel electrophoresis, stained with SYBR Safe stain (Invitrogen, Groningen, The Netherlands) and visualized using the ENDURO GDS Gel Documentation System (LabNet, Edison, NJ, USA). The parental fish were evaluated using the primers for microsatellite regions and for each couple was selected a pair of primers presenting different size of amplicons from male and female parental fish. The primers used to analyze the offspring of R2 and R4 was the primer ASTY 16 (165 pb) and for R3 the primer ASTY 13 (160 pb) (ZAGANINI et al., 2012).

2.7. Sex Ratio and cytogenetic analysis

At eight-months age, fishes obtained from the section 2.4 (R1, R2, R3 and R4) were euthanized using menthol (Êxodo Científica, Hortolândia, Brazil) at 100 mg L^{-1}. Sex ratio was performed by external visualization of gonads. Some fishes died over time due to diseases and number of samples were different among repetitions. Five fish from diploid control and diploid

gynogenetic treatments were used for chromosome analysis by cytogenetic preparations. Mitotic preparations were performed according to (BERTOLLO et al., 1986) and stained with Giemsa solution.

2.8. Statistics

Results are shown as mean ± standard error. The data were checked for normality using the Liliefors test and analyzed by ANOVA. Data expressed as percentages were transformed in order to fit the assumptions of statistical variance homogeneity using the Levene test (BROWN and FORSYTHE, 1974). Analysis was performed using the software STATISTICA v. 10.0 (Statsoft, Tulsa, U.S.A.) and significance was set at $P < 0.05$.

3. Results

3.1. Optimization of UV-irradiation of yellow tetra sperm

Data of developmental stages are described on Table 1. No differences among all treatments were observed for unfertilized eggs (*P = 0.4489*), 2-cell (*P = 0.4489*), blastula (*P = 0.0546*), gastrula (*P = 0.0643*) and hatching stage (*P = 0.0606*). However, in general, the results showed a reduction on survival at the dose of 120 mJ/cm^{-2}, which was significantly lower for somite stage (*P = 0.0468*). No normal larvae were observed in the irradiated treatment and all were abnormal (100%) when compared with the control (2.23 ± 1.15%). In the control group, all individuals were diploid from ploidy analysis (Table 1). Excepted for 2 aneuploid embryos found

in the 40 mJ/cm^{-2} of UV-irradiation, all other embryos in the irradiated groups were haploid (Table 1). Haploid larvae showed the characteristic "haploid syndrome" with several abnormalities, such as shortened body and bent tail. Morphologies of normal (diploid) and abnormal (haploid) larvae are showed on Fig. 1.

Sperm parameters are detailed on Table 2. UV-irradiation significantly decreased (*P = 0.0032*) the sperm motility (%) in the irradiated groups and no difference were observed for VCL (*P = 0.3351*), VAP (*P = 0.1285*) and VSL (*P = 0.1182*). Regarding the sperm viability, all treatments presented higher values, with also no difference (*P = 0.9674*). Based on those results, the UV-irradiation dosage of 80 mJ/cm^{-2} was selected for induced gynogenesis trials.

3.2. Heat shock to induce diploid gynogenesis

Data of developmental stages are described on Table 3. No differences were observed between the treatments for unfertilized (*P = 0.5947*), 2-cell (*P = 0.5947*), blastula (*P = 0.5946*) and gastrula (*P = 0.1678*) stages. For somite and hatching stages, presumptive gynogenetic diploid treatment presented decreased survival (42.69 ± 13.41% and 37.29 ± 10.37%, respectively) in comparison with other treatments (*P = 0.0474* and *P = 0.0093*, respectively), in special with the diploid control (87.27 ± 3.27% and 85.58 ± 3.03%, respectively). At hatching stage, all larvae from the haploid group were abnormal (100%; *P = 0.0001*). However, the presumptive gynogenetic diploid group also present increased percentage of abnormal larvae (65.44 ± 14.33%, *P = 0.0001*) when compared with diploid control (18.52 ± 5.62%).

The analysis of flow cytometry showed that all individuals from haploid group were haploid (1C, presenting the same DNA content observed in spermatozoa from diploid males) (Fig.

2a). For the diploid control (Fig. 2b), the mean DNA content was similar to that observed for presumptive gynogenetic diploid individuals (2C) (Fig. 2c). As expected, the larvae from the group fertilized with non-irradiated sperm and submitted to heat shock were triploids (3C) (Fig. 2d).

3.3. Sex ratios and cytogenetic analysis

As shown in Table 4, sex ratio of gynogenetic individuals from R1, R2 and R3 were predominantly females. Surprisingly, R4 has increased number of males. Additionally, karyotypes of diploid control and gynogenetic diploid individuals revealed 50 chromosomes (Fig. 3).

3.4. Genotyping

Three pairs of microsatellite primers that gave allele-specific band sizes in the parental fish were used to confirm the offspring of *A. altiparanae*. Progenies resulted from irradiated sperm and submitted to heat shock to restore the ploidy were gynogenetic. The DNA bands showed no contribution from the paternal genome in gynogenetic progeny, as illustrated on Fig. 4.

Table 1: Development of yellowtail tetra *Astyanax altiparanae* after sperm uv-irradiation with 0 (control), 40, 80 and 120 mJ/cm^{-2}.

Treatments	Unfertilized (%)	2-Cell (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatch (%)	Normal (%)	Abnormal (%)	Ploidy		
									1n	2n	*
Control	9.66 ± 4.08	90.34 ± 4.08	87.28 ± 3.58	84.04 ± 3.38	81.80 ± 3.37^{a}	78.89 ± 2.88	97.56 ± 1.15^{a}	2.44 ± 0.61^{a}	0	160	0
40 mJ cm^{-2}	11.24 ± 5.00	88.76 ± 5.00	83.88 ± 4.68	80.04 ± 5.32	76.53 ± 4.98^{ab}	73.15 ± 4.67	0.00 ± 0.00^{b}	100.00 ± 0.00^{b}	158	0	2
80 mJ cm^{-2}	15.70 ± 7.14	84.30 ± 7.14	78.63 ± 7.00	75.38 ± 7.49	72.28 ± 7.05^{ab}	69.81 ± 7.03	0.00 ± 0.00^{b}	100.00 ± 0.00^{b}	160	0	0
120 mJ cm^{-2}	33.33 ± 16.56	66.67 ± 16.56	50.26 ± 15.63	48.39 ± 15.34	46.02 ± 14.55^{b}	44.42 ± 14.61	0.00 ± 0.00^{b}	100.00 ± 0.00^{b}	160	0	0
P - value	*0.4489*	*0.4489*	*0.0546*	*0.0643*	*0.0468*	*0.0606*	*0.0002*	*0.0254*	-		

* hyper haploid (abnormal). Different superscript letters denote statistical differences by the tukey test (ANOVA, $P < 0.05$).

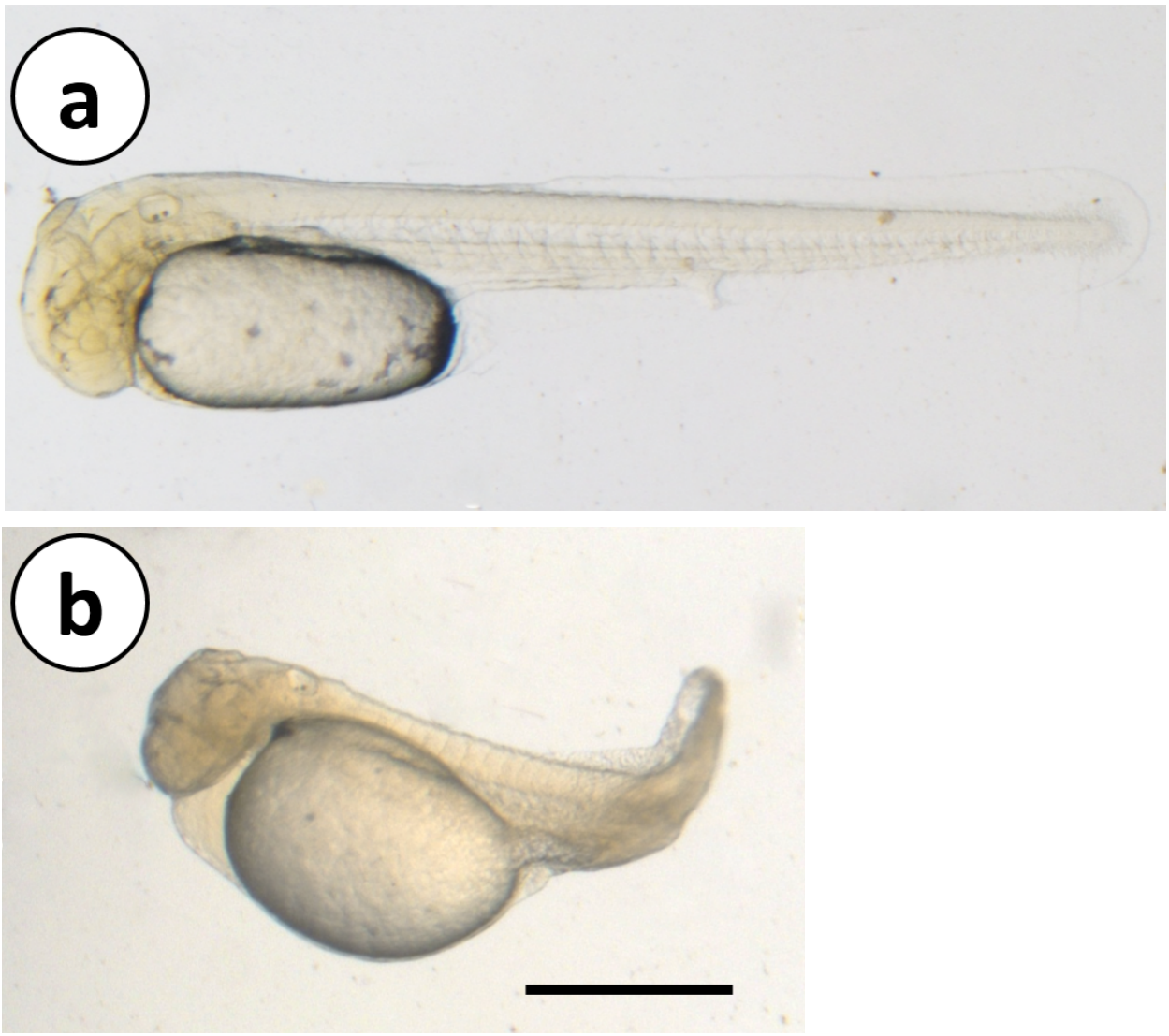

Figure 1: External morphology of *Astyanax altiparanae* larvae. Normal diploid larva from the diploid control group (a); haploid abnormal larva resulted from oocyte fertilized with irradiated spermatozoa (b). Scale: 500 μm.

Table 2: Effects of UV-irradiation on sperm parameters in *Astyanax altiparanae*

Treatments	MOT (%)	VCL (µm s $^{-1}$)	VAP (µm s $^{-1}$)	VSL (µm s $^{-1}$)	Viability* (%)
Control	88.88 ± 2.93^{a}	33.22 ± 5.99	35.11 ± 5.47	33.68 ± 5.19	95.10 ± 0.01
40 mj/cm^2	54.10 ± 11.89^{ab}	31.37 ± 3.58	34.05 ± 4.04	32.70 ± 3.96	95.60 ± 0.01
80 mj/cm^2	34.92 ± 17.58^{b}	29.51 ± 4.53	25.91 ± 2.19	24.56 ± 2.01	95.60 ± 0.01
120 mj/cm^2	12.66 ± 7.50^{b}	22.02 ± 2.84	23.04 ± 3.26	21.83 ± 3.33	96.40 ± 0.01
P-value	*0.0032*	*0.3351*	*0.1285*	*0.1182*	*0.9674*

Different superscript letters denote statistical differences by the tukey test (ANOVA, $P < 0.05$). VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity. * Percentage of live cells.

Table 3: Development of yellowtail tetra *Astyanax altiparanae* after heat- shock treatment for gynogenesis induction

Treatments	Unfertilized (%)	2-Cell (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatch (%)	Normal (%)	Abnormal (%)
Diploid control	6.74 ± 1.98	93.26 ± 1.98	92.51 ± 2.12	90.27 ± 2.66	87.27 ± 3.27^{a}	85.58 ± 3.03^{a}	81.48 ± 5.62^{a}	18.52 ± 5.62^{a}
Haploid group	23.59 ± 13.31	76.41 ± 13.31	74.64 ± 13.66	71.50 ± 13.89	66.75 ± 12.98^{ab}	61.98 ± 11.55^{ab}	0.00 ± 0.00^{c}	100.00 ± 0.00^{c}
Gynogenetic diploid	21.16 ± 11.36	78.84 ± 11.36	75.75 ± 11.99	56.97 ± 12.66	42.69 ± 13.41^{b}	37.29 ± 10.37^{b}	34.56 ± 14.33^{b}	65.44 ± 14.33^{b}
Triploid control	8.95 ± 4.56	91.05 ± 4.56	88.18 ± 6.49	82.73 ± 7.65	76.08 ± 8.93^{ab}	74.87 ± 8.23^{ab}	57.09 ± 16.38^{ab}	42.91 ± 14.65^{ab}
P-value	*0.5947*	*0.5947*	*0.5946*	*0.1678*	*0.0474*	*0.0093*	*0.0001*	*0.0001*

Different superscript letters denote statistical differences by the tukey test (ANOVA, $P < 0.05$).

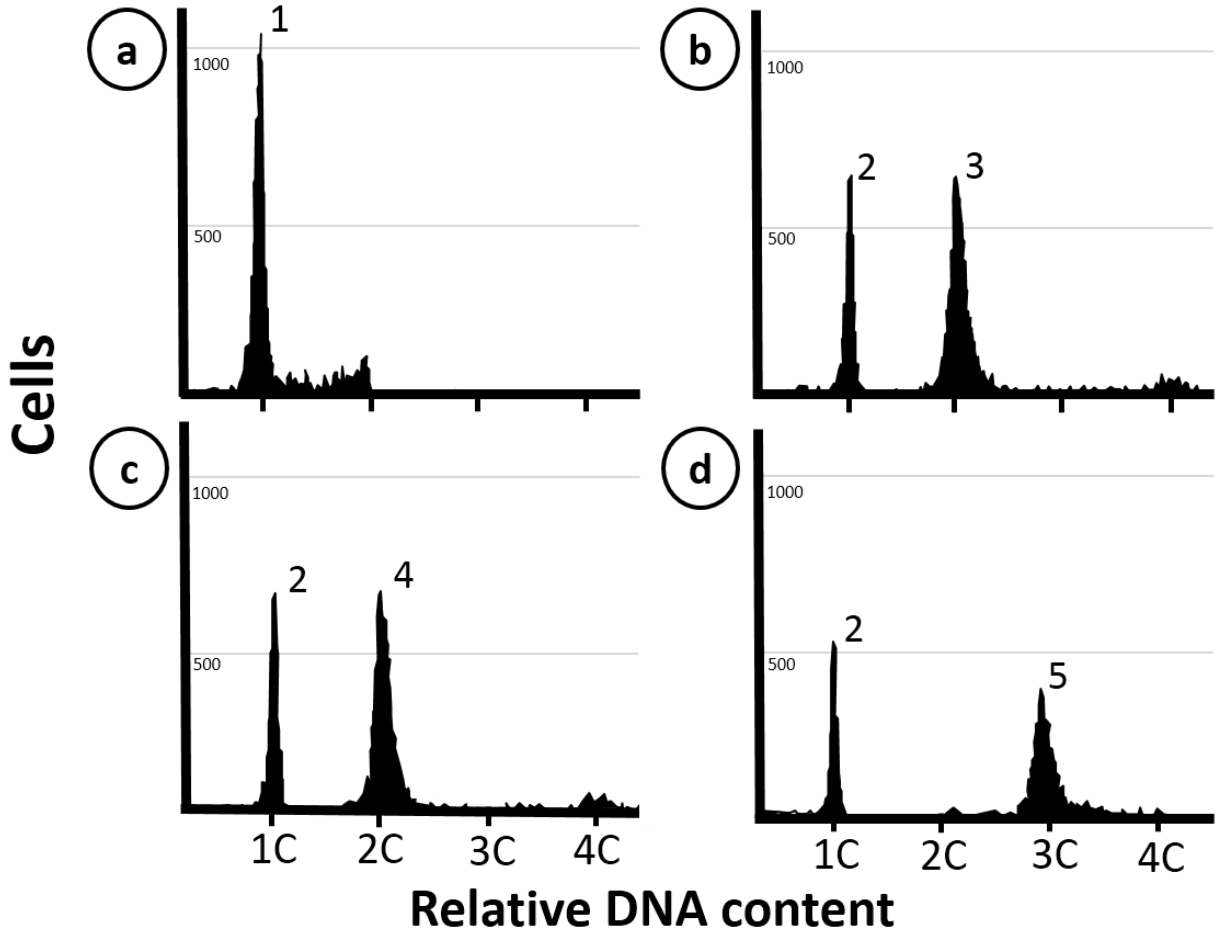

Figure 2: Flow cytometry histogram showing relative DNA content of the haploid group (a), diploid control group (b), presumptive diploid ginogenetic (c) and triploid control individuals (d). 1: haploid group; 2: spermatozoa (haploid); 3: diploid control; 4: gynogenetic diploid and 5: triploid control.

Table 4: Sex ratios of diploid control and gynogenetic diploid *of Astyanax altiparanae*

Repetitions	Sex			
	2n Control		2n Gynogen	
	Female	Male	Female	Male
R1	9	13	4	0
R2	17	13	10	0
R3	26	11	49	0
R4	10	21	11	19

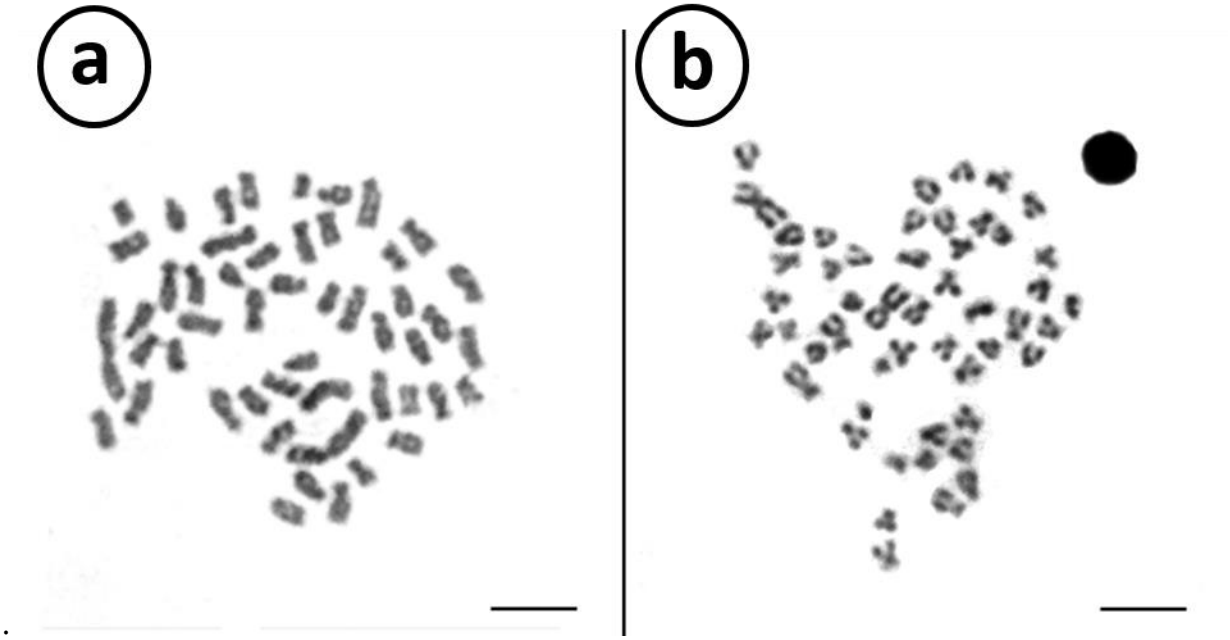

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Figure 3: Metaphase chromosomes of diploid control (a) and gynogenetic diploid (b) individuals of yellowtail tetra *Astyanax altiparanae* showing the presence of 50 chromosomes. Scale: 10 µm

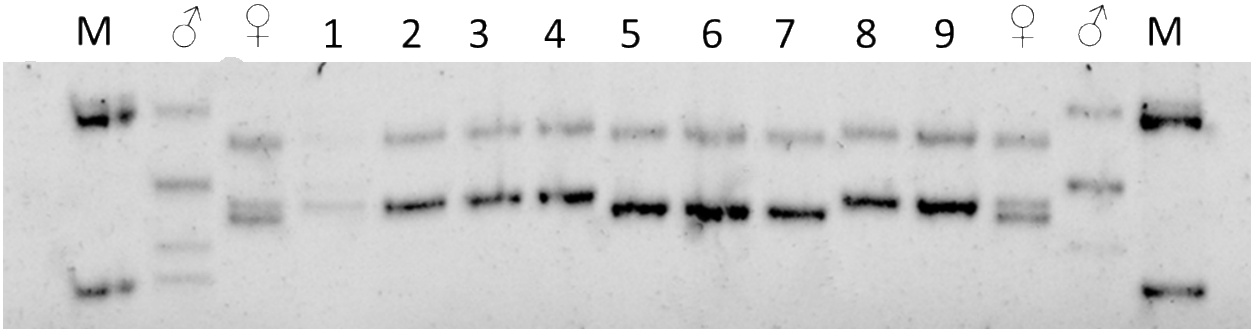

Figure 4: Representative microsatellite electrophotogram of gynogenetic *Astyanax altiparanae* induced by UV-irradiated sperm and heat shock. M: DNA marker; ♀: maternal; ♂: paternal. 1-9: progenies of gynogenesis. Samples prevenient from repetition 4 (section 2.4) using the primer Asty 16 (165 bp) (ZAGANINI et al., 2012).

4. Discussion

In the present study, it was developed the first protocol to induce diploid gynogenesis in a Neotropical fish using UV-irradiation in spermatozoa of *A. altiparanae* and heat shock to restore the diploid status. Optimization of UV-irradiation is the first and essential step for the induction of diploid gynogenesis (PIFERRER et al., 2009) and requires inactivation of sperm genome that can also maintain the fertilization capacity (DIETRICH et al., 2005).

In our results, the UV-irradiation did not affect any parameter of spermatozoa velocity and membrane integrity. Such results are important, since velocity and membrane integrity are essential for fertilization (HONEYFIELD and KRISE, 2000; BOSCHETTO et al., 2011). On the other hand, UV-irradiation leads to a progressive reduction in sperm motility (%), which are expected and commonly observed in other species, such as Atlantic cod *Gadus morhua* (WHITEHEAD et al., 2012) and Rainbow Trout (*Oncorhynchus mykiss*)(DIETRICH et al., 2005). Therefore, by establishing an optimal UV-dosage, irradiated spermatozoa potentially maintain its fertilization capacity, as also described in several studies (PAN et al., 2017). In the spermatozoa, UV-irradiation causes DNA fragmentation (DIETRICH et al., 2005) which can lead to no paternal genetic contribution to the offspring. Our results showed that among the dosages evaluated, aneuploid individuals were observed in the treatment with 40 mJ/cm^{-2}, which suggest that such dosage were lower and failed in fully inactivate the paternal genome. On the other hand, the dosage of 120 mJ/cm^{-2} significantly affect the capacity of the sperm to support embryo development, leading to lower survival. Therefore, the dosage of 80 mJ/cm^{-2} was chosen because it was efficient to inactivate the paternal genome without significantly compromise the sperm motility, fertilization ability and embryo survival.

The inactivation of the paternal genome without restoring the diploidy leads to all haploid and abnormal larvae, which indicates that the sperm were completely inactivated. This haploid condition is well-known as "haploid syndrome" in which the larvae present several abnormalities and die shortly after hatching (TVEDT et al., 2006; PAN et al., 2017). Therefore, for gynogenetic induction, the diploid condition must be restored by generally applying thermal or pressure shocks (KOMEN and THORGAARD, 2007), as also observed in our studying species.

In our study, the same heat shock procedures used by (ADAMOV et al., 2017) for triploid induction in *A. altiparanae* was used successfully for diploidization. Consequently, we obtained gynogenetic meiotic and triploid individuals by retention of the second polar body (ADAMOV et al., 2017). Meiotic gynogenesis is an interesting approach to produce homozygotes in fish since present higher survival than mitotic gynogenesis (KOMEN and THORGAARD, 2007) as it was demonstrated by other studies (ARAI, 2001; JIANG et al., 2017). Furthermore, the survival rate of meiotic gynogenetic individuals seems to increase in further generations, which could be related to the decrease of recessive lethal genes (JIANG et al. 2017).

Gynogenesis can also be used to investigate the sex determination system (KOMEN and THORGAARD, 2007). In the present study, three repetitions had sexual proportions closely to 100% females. However, one group presented higher amounts of males. Such results provide evidence that *A. altiparanae* is a species with predominance of female homogamety (female – XX, male – XY). Therefore, besides all fish were reared in the same conditions, the presence of males suggests that other factors, such as environmental influences on the sex reversal of genetic females to physiological males. Additionally, autosome or polygenic loci could be also important in sex determination of *A. altiparanae*. This knowledge are also interesting since females grows faster and are larger than males (GARUTTI, 2003) and future protocols for the direct or indirect sex

control (LUCKENBACH et al. 2017) could be applied in the establishment of monosex female production of *A. altiparanae*.

The gynogenesis in *A. altiparanae* was also confirmed by microsatellite analysis, which is an accurate analysis and are generally accomplished in other species, such as the yellow drum *Nibea albiflor* (Chen et al. 2017). The selection of specific primers for each couple was important to ensure different patterns of bands and the results showed that gynogenetic individuals analyzed inherited only maternal alleles, which suggest exclusively maternal genetic transmission. Cytogenetic analysis also showed the presence of 50 chromosomes, confirming the results of flow cytometry that gynogenetic individuals are diploid. Therefore, the combined methods (e.g. flow cytometry, microsatellite markers and cytogenetic), with each presenting a particular advantage, confirms the efficacy of our protocol.

Therefore, in this study an efficient protocol for meiotic induction of gynogenetic was established for the first time in a Neotropical species. Such a protocol was optimized using the following parameters: 80 mJ/cm^{-2} of UV-irradiation for sperm inactivation; diploidization with heat shock (40°C; 2 min) 2 minutes post-fertilization. Such a protocol gave rise to predominantly females, suggesting a XX-XY sex determining system. Additionally, the data are innovative and applicable for generation of monosex and clonal lineages for applied and basic sciences.

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Considerações finais

O presente trabalho mostrou importantes e inéditas informações na área de manipulação cromossômica em espécies nativas, em particular, peixes tetraploides e ginogeneticos. O protocolo de tetraploidização foi muito eficiente, com 94,55% de larvas tetraploides. Na literatura, estudos focados na obtenção de peixes tetraploides são escassos e limitados a poucas espécies, sendo comumente relatado pelos autores como uma tarefa de difícil concretização, o que evidencia a importância deste estudo. Além disso, a obtenção de peixes adultos poderá viabilizar outros estudos de manipulação cromossômica, como produção em massa de peixes triploides e protocolos de androgênese e ginogênese. Portanto, a viabilidade dos gametas de tais indivíduos deverá ser futuramente analisada.

Este trabalho também mostrou a possibilidade de se obter peixes ginogenéticos por meio da fertilização com espermatozoide irradiado e choque de temperatura para restauração da diploidia. A ginogênese é uma ferramenta interessante para o estabelecimento de linhagens monosexuais femininas e clonais, além de ser usada para avaliar o mecanismo genético de determinação sexual. Além disso, este protocolo será o primeiro da região Neotropical, e provavelmente servirá de base para futuros estudos com outras espécies nativas.

Portanto, destacam-se os interessantes resultados e a possibilidade de aplicação na produção em curto prazo. Além disso, servirão como base para similares estudos em outras espécies nativas.