

Research Article

The sterile male release approach as a method to control invasive amphibian populations: a preliminary study on *Lithobates catesbeianus*

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Editor's note:

This study was first presented at the 19th International Conference on Aquatic Invasive Species held in Winnipeg, Canada, April 10–14, 2016 (<http://www.icaais.org/html/previous19.html>). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Widespread populations of the invasive species *Lithobates catesbeianus* (American bullfrog) are present in different parts of the world and are difficult to control. This study investigated the possibility to sterilize male individuals of this species in order to use the sterile male release technique in controlling these invasive populations. The technique can be adopted in aquaculture facilities as well to prevent new introductions by incidental releases or escapes. In order to produce sterile individuals a cold and pressure shock protocol were used on fertilized eggs to create triploid individuals. The cold shock did not result in triploid individuals while 54% triploids were obtained from pressure shock, the remaining individuals being aneuploid. The triploid and control larvae were reared for more than one year and the control larvae had a greater length after 7 and 9 months, their weight did not differ. At metamorphosis no difference in length and weight was found between the two groups. The subadult bullfrogs from both groups showed 9 months post metamorphosis a similar scaled body mass index but lower than individuals living in the wild. The pressure shock protocol needs further refinement in respect to the timing of the shock. Further research on the reproductive behavior of these sterile frogs is necessary to evaluate the possible use in the sterile male release technique.

Key words: hydrostatic shock, invasive species, *Rana catesbeiana*, sterile male release technique, cold shock, triploidy

Introduction

Invasive alien species are considered one of the five major threats to biodiversity (Secretariat of the Convention on Biological Diversity 2006). The importance of this issue is recognized in the recently published EU Regulation (EU 1143/2014) on Invasive Alien Species (IAS) (European Parliament and Council of the European Union 2014). Apart from stopping new introductions, member states are obliged to control

recently established invasive populations of listed species, such as the American bullfrog (*Lithobates catesbeianus* Shaw, 1802). Currently, the traditional methods applied to control population size of IAS are focusing on direct techniques to reduce the numbers of individuals. Often these measure have no effect on the reproduction or population growth rate (Govindarajulu et al. 2005).

Indirect control techniques are an option to control invasive populations. In this respect changing

the sex ratio by introducing sterile males in the population, eventually enhanced with pheromones attraction, or the introduction of genetically altered individuals with inserting fatal genes are potential techniques (Bergstedt and Twohey 2007; Louette 2012). In insects, the sterile male release technique (SMRT) provides an effective reduction in population size in proportion to the ratio of sterile to fertile males (Krafsur 1998). This technique can be very effective if males have the monopoly over one or multiple females and if every sterile male is truly competitive with the fertile male individuals (Gonçalves da Silva et al. 2010; Knipling 1959; Lance and McInnis 2005). The sterilizing agent should induce over 90% sterility in males and environmental conditions have to be more or less constant (Knipling 1959). To completely eradicate a population sterile males still have to be introduced even when the population appears to be extinct (Knipling 1959; Krafsur 1998).

The first study of the sterile male release technique using chemical sterilization was successfully implemented on the screwworm fly in 1966 (Baumhover 1966) and other insects, such as the fruit fly (*Ceratitis capitata* Wiedemann, 1824), tsetse fly (*Glossina* spp.) and pathogenic mosquitos (Klassen and Curtis 2005). The SMRT through chemical sterilization was also used for the sea lamprey (*Petromyzon marinus* Linnaeus, 1758) in an attempt to control the invasive population in the Laurentian Great Lakes, US (Bravener and Twohey 2016; Hanson and Manion 1980; Twohey et al. 2003).

Sterilization can be carried out with chemicals such as bisazir or implants of D-Arg GnRH (Klassen and Curtis 2005; Young et al. 2004). The use of less toxic chemicals such as nonoxynol-9, benzalkonium chloride, cholic acid, gossypol acetic acid, tannic acid, zinc acetate, cysteamine, propranolol and cupric chloride have been tested on sea lamprey. None of these agents was as effective as the hazardous bisazir (Ciereszko et al. 2004; Rinchard et al. 2000). Besides chemical agents, physical methods can also be adopted to sterilize animals. Exposure to UV radiation resulted in DNA fragmentation and a lower fertilization speed in sperm of sea lampreys and rainbow trout (*Salmo gairdneri* Walbaum, 1792) and a failure in development in sea urchin embryos (*Sphaerechinus granularis* Lamarck, 1816) (Ciereszko et al. 2005; Dietrich et al. 2005; Pruski et al. 2009). Gamma radiation of Cobalt-60 or Cesium-137 and X-ray radiation have been applied on gambas, crayfish and sea lamprey, resulting in a reduced reproductive success but less effective as the use of chemosterilantia (Aquiloni et al. 2014; Hanson 1990; Sellars et al. 2005).

Physical inductors are a promising technique to obtain sterility by inducing triploidy. Allotriploid and autotriploid individuals in lower vertebrates can sporadically occur in nature through parthenogenesis, gynogenesis and hybridogenesis. Triploid animals are generally sterile due to pairing problems in meiosis that delivers unbalanced gametes (Bradshaw 1992). Triploidy can be artificially induced by a cold, heat or pressure shock. During early embryonic development these shocks block the extrusion of the second polar body in the second meiotic division, which results in a triploid zygote. One set of chromosomes will be paternal, while two sets are maternal (Peruzzi and Chatain 2000).

Artificial production of triploid larvae by a cold shock on the fertilized eggs was first performed in the newt *Notophthalmus viridescens* (Rafinesque, 1820) (Fankhauser and Griffiths 1939). Heat shock treatment of eggs of the rainbow trout gave a range from 10–100 % triploid individuals depending on the temperature and time of treatment. Histological analysis showed sterility of the gonads (Solar et al. 1984). The effectivity of temperature shocks to produce triploids varies widely (Benfey and Sutterlin 1984; Ferrier and Jaylet 1978; Ojima and Makino 1978; Solar et al. 1984) and the choice for cold or warm shocks might depend on the temperature for normal development of the embryos but both heat and cold shocks can result in triploids in some species (Hussain et al. 1991; Linhart et al. 1991; Recoubratsky et al. 1992).

In aquaculture, where artificial production of triploids is applied on a large scale, the use of pressure shock is recognized as the most efficient and economical method to produce sterile fishes (Benfey 2001; Cassani and Caton 1986; Huergo and Zaniboni-Filho 2006; Peruzzi and Chatain 2000; Preston et al. 2013). In this branch the technique is applied to obtain a higher growth, survival and flesh quality due to the inhibition of sexual maturation (Krasznai and Marian 2006). The time after fertilization, the amount of pressure applied on the eggs and the duration of the shock are important variables that will determine the outcome of the triploid yield.

The American bullfrog is native to eastern North America, extending from south-eastern Canada south to central Florida and north-eastern Mexico (IUCN SSC Amphibian Specialist Group 2015). This extended natural range is related to the species' flexible life history and broad climatic and ecological tolerance, which contribute to its success in invading new habitats (D'Amore 2012).

Invasive American bullfrog populations are present in several continents throughout the world: Africa, Asia, Europe, North-America and South-America (CABI 2016) where it forms a threat to native

biodiversity through predation, habitat and food competition (Adriaens et al. 2013; Batista 2002; Blaustein and Kiesecker 2002; Cross 2002; Wang and Li 2009). This species is a vector of the chytrid-fungus (*Batrachochytrium dendrobatidis* Loncore, 1999) (Blaustein and Kiesecker 2002; Hanselmann et al. 2004; Pasmans and Martel 2012). This fungus causes chytridiomycosis, an infectious disease considered as one of the main causes of global amphibian decline and extinction (Bellard et al. 2016). It is favored by climate change that shifts the temperature toward the growth optimum of this fungus, thereby encouraging outbreaks (Garner et al. 2006; Pounds et al. 2006). Consequently, the American bullfrog has been considered one of the world's 100 worst invasive species, based on its invasive character and ecological impact (Lowe et al. 2000).

Control methods for the American bullfrog are limited to the direct removal of larvae and adults by the use of fyke netting traps, shooting or draining the ponds they inhabit. However, these measures have proved to be insufficient to prevent the further expansion of populations (Adriaens et al. 2013; Berroneau et al. 2008; Devisscher et al. 2012; Mandin 2015; Rosen and Schwalbe 1995). Successful eradications in Europe have only been achieved at an early stage of invasion (Banks et al. 2000; Ficetola et al. 2007; Theismeier et al. 1994). As such, new control methods such as the sterile male release approach are urgently needed to prevent further spread and negative impacts on native species.

Since the use of chemicals such as bisazir can pose an environmental threat to aquatic ecosystems (Descamps and De Vocht, unpublished results; Rudrama Devi and Reddy 1985; U.S. Fish and Wildlife Service 1995), the use of chemical sterilization is not an option to sterilize male bullfrogs. Besides that, the European legislation (Directive 2001/18/EC) prevents the release of transgenic individuals (European parliament and Council of the European Union 2001). The barriers to use chemical sterilants and the potential non-target effects of releasing transgenic individuals in the native range is the main reason why this preliminary research focuses on the production of triploids through temperature or pressure shocks and at the same time determine the effect of the treatment on their growth and development. The induced sterility in male and female bullfrogs could be used in a sterile release program. Furthermore, sterile individuals can be used in aquaculture to prevent the establishment of novel non-native populations at the site as many of the introductions are the result of intentional or incidental releases from breeding facilities (Akmentins and Cardozo 2010; Jennings and Hayes 1985).

Methods

Collection, fertilization and treatment

Two males and one female adult American bullfrog were caught using electrofishing (Deka 3000 Lord) and fykes (0.8 m diameter, 1 cm mesh) in June 2014 in a pond in the valley of the Grote Nete, Belgium (51°8'N; 05°8'E). During the experimental procedures each frog was held in a plastic 84 L box with half land and half water biotope at a temperature of 20 °C according to the international, national and institutional ethical standards. The frogs were fed daily with topmouth gudgeons (*Pseudorasbora parva* Temminck and Schlegel, 1846) and bullfrog larvae. The female was injected intraperitoneally with a primary dose of 1 IU/g hCG (Chorulon, Intervet) to induce egg ovulation and 0.1 µg LHRH/g ((Des-Gly¹⁰, D-His(Bzl)⁶, Pro-NHEt⁹)-LHRH, Bachem) to reach egg maturation. Twenty-four hours later an ovulatory dose of 10 IU/g hCG + 1 µg LHRH/g was injected. Forty-eight hours after the first hormone administration matured eggs were collected by kneading the lateral abdomen of the female frog gently while protracting the legs to the head. The two male bullfrogs were euthanized with MS-222, their testes dissected and macerated in SAR (Simplified Amphibian Ringer) solution at pH 7.4 and kept on ice.

After activating the sperm with dechlorinated tap water at 20 °C and checking the motility using an inverse microscope (Olympus CKX41) the gametes were mixed during 10 minutes. After rinsing the eggs with dechlorinated tap water one third of the egg volume was transferred to a high-pressure chamber (Pentair Aquatic Ecosystems, Inc.) where a 6 minutes long pressure of 5000 psi was applied. Another third was transferred to a climate chamber (Binder) for 5 minutes in water of 3 °C. The variables of the temperature and pressure shock were chosen after a literature review on the results of these techniques on amphibian embryos (Table 1 and Table 2). The last third of the egg clutch was used as a control group. After the pressure shock and the temperature shock the experimental and control groups were transferred to 5 L tanks at 22 °C in a biological aquasystem (Fleuren & Nooijen) to develop.

Rearing larvae and subadults

Young larvae (< 5 months) were fed daily with *Spirulina*, older larvae (> 5 months) were fed with Novofect tablets (JBL). The larvae were housed at a density of 2 individuals per liter at 24 °C and a day/night cycle of 12/12.

After metamorphosis the subadults were housed with 10 individuals in an amphibian housing system

Table 1. Overview of variables used in hydrostatic pressure shocks to induce triploidy in amphibia.

| Species | Pressure (psi) | Time after fertilisation (min) | Duration pressure shock (min) | Effectiveness (% triploidy) | References |
|----------------------------------------------|-------------------|--------------------------------|-------------------------------|-----------------------------|-------------------------------------|
| <i>Ambystoma mexicanum</i> (Shaw, 1789) | 10000 | 60 | 8 | 97 | Gillespie and Armstrong 1979 |
| <i>Pleurodeles waltl</i> (Michahelles, 1830) | 6527 | 0–60 | 6 | 95 | Ferrier and Jaylet 1978 |
| <i>Rana pipiens</i> (Schreber, 1782) | 5000 | 5 | 6 | 85 | Dasgupta 1962 |
| <i>Xenopus laevis</i> (Daudin, 1802) | 4800 5878/6613 | 5–10 5 | 6 5 | 100 97 | Tompkins 1978 Müller et al. 1978 |
| <i>Lithobates catesbeianus</i> (Shaw, 1802) | 5000 | 10 | 6 | 54 | Own data |

Table 2. Overview of variables used in temperature shocks to induce triploidy in amphibia.

| Species | Temperature (°C) | Time after fertilisation (min) | Duration cold shock (min) | Effectiveness (% triploidy) | References |
|-----------------------------------------------------|------------------|--------------------------------|---------------------------|-----------------------------|-------------------------------|
| <i>Cynops ensicauda</i> (Hallowell, 1860) | 1.5–2.5 | 1 | 300 | 45 | Fankhauser et al. 1942 |
| <i>Notophthalmus viridescens</i> (Rafinesque, 1820) | 0.5–3 | 1 | 960–1560 | 69 | Fankhauser and Griffiths 1939 |
| | 0–3 | 1 | 300 | 80 | Griffiths 1941 |
| | 34.2–37.2 | 1 | 5–50 | 79 | Fankhauser and Watson 1942 |
| <i>Pleurodeles waltl</i> (Michahelles, 1830) | 37.1 | 1 | 5 | 95 | Ferrier and Jaylet 1978 |
| <i>Lithobates catesbeianus</i> (Shaw, 1802) | 3 | 10 | 5 | 30 | Own data |
| | 3 | 10 | 5 | 0 | Own data |

of 50 × 65 × 90 cm (height × weight × depth) with a one third aquatic part with a depth of 15 cm. The water temperature was kept at 23 °C by a thermostat (Sera). Light was provided with a 13 W UVA/UVB lamp (Exo Terra) in a light/dark regime of 12 h/12 h. A 50 W IR lamp (Exo Terra) was permanently on so the frogs could warm up if needed. In the first months food was supplied in form of crickets and buffalo worms enriched with vitamin and mineral supplements (Trixie) for amphibia. Larger individuals were fed with earthworms, snails, topmouth gudgeons and juvenile *Dubia* roaches (*Blaptica dubia* Serville, 1838).

The length of the larvae was measured (snout-vent length) at 4, 7 and 9 months past fertilization to evaluate the difference in growth between the groups. In fish, growth has often been found alike in triploids and diploids (Maxime 2008) but no data on amphibians are available. After metamorphosis weight and length were evaluated at a 2–3 monthly interval. To compare the body condition of the reared subadult bullfrogs with those living in a natural environment the Scaled Mass Index M_i (Peig and Green 2009) of each group was calculated and compared with those of a dataset of wild caught bullfrogs (n = 57) in the valley of the Grote Nete.

The young metamorphosed frogs that were housed at a density of 10 individuals were susceptible to cannibalism. Although they were ordered by size, the smallest individuals were predated. During the months the density was lowered to 5 individuals/

housing system and after 18 months post fertilization 14 individuals of the control group and 16 of the pressure group were kept at a density of 1 individual per 80-litre box until they reached sexual maturity.

Statistical analyses were performed with SPSS statistics 22 (IBM) using ANOVA and a post-hoc Tukey test for the analysis of the length and weight of the larvae and metamorphosed frogs. Differences were significant when P-values were < 0.05.

In order to test the hypothesis if there is a significant difference in the Beta weights of the linear regression of the reference weight of the wild, control and the pressure subadult group, the corresponding 95% confidence intervals were estimated bias corrected bootstrap (1,000 resamples) (Cumming 2009).

Determining ploidy level

All the larvae of the three groups (C (control): n = 58, T (cold shock): n = 34, P (pressure shock): n = 58) were subjected to determination of the ploidy level at an age of 8 months by the use of a flowcytometric analysis. The larvae were anesthetized with MS-222. A 29 G insulin syringe (Terumo) was filled with 10 µl of heparin and approximately 40 µl of blood was taken out of the heart of the larvae. The blood with heparin was transferred to 1 ml phosphate buffered saline (PBS) on ice. The solution was centrifuged twice during 5 min. at 4 °C (800 rpm). Between the 2 centrifuges the PBS was removed and the blood

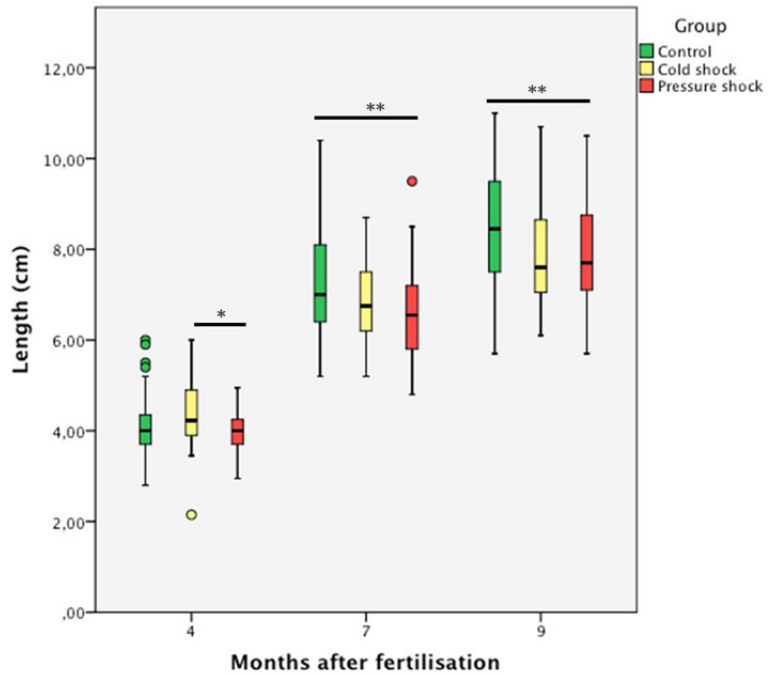


Figure 1. Overview of the larval length after 4, 7 and 9 months of development (* $p < 0.05$, ** $p < 0.01$).

pellet was resuspended with 1 ml ice-cold PBS and 5 μ l heparin. 50 μ l of cell suspension was added to 450 μ l 0.1% TritonX-100. Two hundred μ l of this suspension was added to 10 μ l propidium iodide and incubated in the dark during 15 minutes. The cell suspension was mixed with a control diploid bullfrog cell suspension and analyzed with a FACSCalibur (BD Biosciences) flow cytometer, counting 10,000 events. Flowcytometric analysis was performed with Cellquest Pro (BD Biosciences). Based on the results of the flowcytometric analysis only the control and pressure shock group were kept to grow until sexual maturity.

Results

Larval and subadult rearing after the experiments

One hundred and twenty four larvae developed in the aquasystem (control group $n = 61$, cold shock group $n = 42$, pressure shock group $n = 61$). Four months post fertilization the control group had a mean \pm SD length of 4.09 ± 0.67 cm, the cold shock group 4.38 ± 0.75 cm and the pressure shock group 4.02 ± 0.50 cm (Figure 1). Length was significantly different between the groups ($F_{2,161} = 4.33$, $p = 0.015$). The post-hoc test indicated that length of the cold shock group was significantly larger than the pressure shock group ($p = 0.014$). No significant difference in length was found between the control and cold shock group

($p = 0.060$) and the cold shock versus pressure shock group ($p = 0.812$).

After 7 months the control group had a mean \pm SD length of 7.26 ± 1.22 cm, the cold shock group 6.86 ± 0.95 cm and the pressure shock group 6.60 ± 0.94 cm (Figure 1). Length after 7 months significantly differed between groups ($F_{2,161} = 6.06$, $p = 0.003$). Length of the control group was significantly higher compared to the pressure shock group ($p = 0.002$). No significant difference was found between the control and cold shock group ($p = 0.145$) and the cold versus pressure shock group ($p = 0.433$).

Two months later the control group had a mean \pm SD length of 8.49 ± 1.32 cm, the cold shock group 7.93 ± 1.16 cm and the pressure shock group 7.95 ± 1.12 cm (Figure 1). Length after 9 months significantly differed between groups ($F_{2,161} = 4.06$, $p = 0.019$). Length of the control group was significantly higher compared to the pressure shock group ($p = 0.037$). No significant difference was found between the control and cold shock group ($p = 0.051$) and the cold versus pressure shock group ($p = 0.990$).

The first larvae from the control group metamorphosed 272 days post-fertilization, while the first individuals from the cold and the pressure shock group metamorphosed after 316 and 307 days respectively. A small number of individuals (5% and 8% of respectively pressure shock and control group) metamorphosed only after 610 days post fertilization.

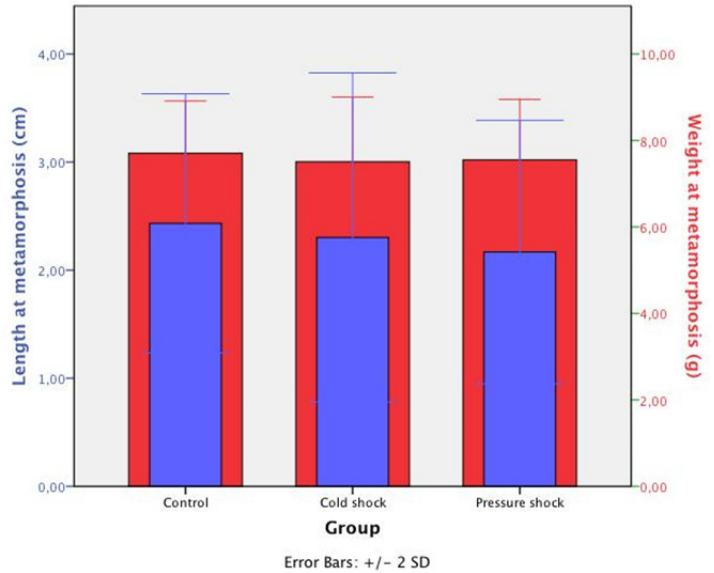


Figure 2. Weight and length at metamorphosis of the control, cold and pressure shock group.

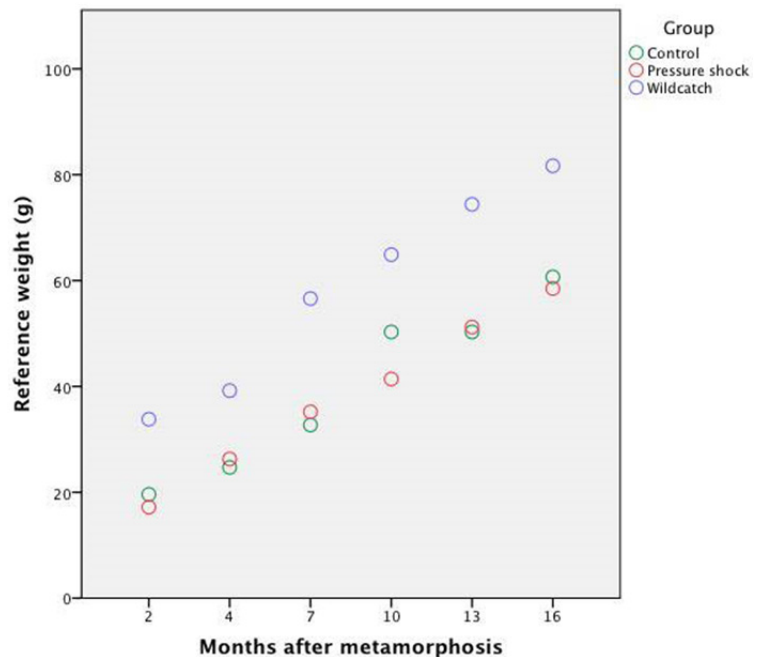


Figure 3. Results of the Scaled Mass Index for wild caught (W), raised control-diploid (C) and pressure shock -triploids (P) subadult frogs.

At metamorphosis the subadults from the control group had a mean \pm SD length (snout-vent) of 3.07 ± 0.25 cm. The cold and pressure shock group reached a mean \pm SD length of 3.00 ± 0.30 and 2.99 ± 0.27 cm respectively (Figure 2). There was no significant difference in length between the groups ($F_{2,147} = 1.17$, $p = 0.315$). The subadults from the control group had a mean \pm SD weight of 6.07 ± 1.51 g, the cold and pressure shock group reached a weight of 5.76 ± 1.90 and 5.39 ± 1.55 g respectively (Figure 2). No signi-

ficant difference was found in weight between the groups ($F_{2,147} = 2.49$, $p = 0.086$).

Scaled Mass Index of the control and pressure shock reared bullfrogs was lower compared to the wild bullfrogs (Figure 3). As the 95% confidence intervals fully overlap, the beta weights are not considered statistically significant different from each other ($p > 0.05$). The maximal gain in weight and length of the reared frogs at 8 months post metamorphosis was 85.92 g and 69.2 mm.

Determining ploidy level

The flowcytometric analysis showed that the individuals of the control and the cold shock group were all diploid. In the pressure shock group 46% of the individuals was aneuploid and 54% triploid.

Discussion

The use of a pressure shock to induce triploidy in American bullfrogs was found to work. Applying a pressure shock on fertilized eggs of the American bullfrog was found to be a more accurate method to induce triploidy than the use of a cold shock. During trial experiments the cold shock protocol resulted in 30% to 0% triploids. The lack of triploidy in this study after cold shock in combination with a high variability in triploidy during trial experiments confirms the statements in literature that the outcome of applying temperature shocks on fertilized eggs is not as solid compared to the application of hydrostatic pressure (Cassani and Caton 1986; Gillespie and Armstrong 1979; Gillet et al. 2001; Hussain et al. 1991). Although the pressure shock resulted in triploid individuals, the outcome of the experiment did not achieve a high yield in triploids (54%). Studies on the production of triploids in amphibians with pressure shocks obtained a triploid yield from 85–100% (Dasgupta 1962; Ferrier and Jaylet 1978; Gillespie and Armstrong 1979; Müller et al. 1978; Tompkins 1978). Our results show that the pressure intensity applied in this protocol is sufficient to induce triploidy but that other variables as time after fertilization and duration of the shock have to be optimized. This will make sure that the secondary polar body stays in the zygote so that the embryo is triploid and a maximal production of triploids in bullfrogs can be obtained.

The growth and size at metamorphosis of the larvae in laboratory conditions varied slightly among the different groups. During the approximately 9 months as a larvae, the individuals of the pressure shock group were significantly smaller than the others. These differences in growth were neutralized at metamorphosis where the subadults from the 3 groups did not differ significantly in length and weight. This could cause a problem when these smaller triploid larvae would be released in the wild for application in SMRT. Smaller individuals potentially will be faster preyed upon resulting in a decrease of their abundance. According to the results of this study it would be beneficial to release the triploids after metamorphosis to avoid size differences that could give a disadvantage in predation or mating since larger male individuals have a higher chance to control an oviposition site and therefore mating with a female (Emlen 1976).

The larvae that were reared in this study metamorphosed in general at a smaller length compared to individuals in natural conditions (Ryan 1953). This can be due to the higher temperature in laboratory condition and hence a shorter time to metamorphosis. A difference of forty-four days was recorded between the start of the metamorphosis of the diploid and triploid/aneuploid animals. The growth of the diploid and triploid subadult frogs in this study is comparable to the growth in a moderate climate during the favorable seasons (Bruneau and Magnin 1980; Raney and Ingram 1941; Ryan 1953). Bullfrogs cultured in outdoor pens with an air temperature from 25 °C to 30 °C had an average weight of 175 g eight months post metamorphosis (Lutz and Avery 1999). In our study subadult bullfrogs raised at a temperature of 23 °C gained up to 69.2 mm in length and 85.92 g in weight during the 8 months post metamorphosis. This growth is higher than stated for wild caught animals in a moderate climate but only half of the ones cultured for food in tropical conditions. The result reflects the fact that our bullfrogs had a continuous growing season but at a lower temperature than available in tropical regions.

The Scaled Mass Index reveals that individuals found in the wild in the Valley of the Grote Nete have a higher weight for a specific reference length compared to those bred in laboratory condition. In this study the rearing of subadult bullfrogs was very delicate. The animals suffered from stress and had difficulties in feeding. The lack of appropriate environmental factors as available sunlight, shelter and an appropriate diet can also be considered a cause for the difference in weight. To release metamorphosed triploids for application in SMRT it is essential to mimic the natural conditions as much as possible to end up with a M_i similar to the wild population so that competition for resources, females and probability of predation is equal.

This study is the first of his kind for the American bullfrog to show that applying pressure shocks on embryos is a promising technique to produce sterile individuals. Successful production of triploid amphibian embryos has been reported in literature (Dasgupta 1962; Fankhauser et al. 1942; Fankhauser and Griffiths 1939; Ferrier and Jaylet 1978; Gillespie and Armstrong 1979; Griffiths 1941; Kawamura 1941). Despite studies on embryology, triploid amphibians have never been reared for the purpose of creating sterile individuals to be released in a sterile male program. However, to avoid the pitfalls experienced by other SMRT programs (Alphey et al. 2010; Bravener and Twohey 2016) more work is needed before full scale implementation in the field can occur. When the reared diploid (control) and triploid (pressure shock)

bullfrogs reach sexual maturity their behavior, foraging and sexual activity will be studied and compared with wild bullfrogs (Descamps and De Vocht 2016). Therefore, an accurate estimation of the size of the target population bullfrogs and their population structure will have to be made. Moreover, mathematical models will have to clear out at what stage and at what amount it is favorable to release the triploid tadpoles so that survival, population reduction and cost-efficiency is maximal and optimal. Further research to optimize the effectivity of different trap systems have to be developed in order to enhance the catchability of adult bullfrogs.

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Ethical approval: All applicable national and institutional guidelines for the care and use of animals were followed (approval number 201024).

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