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Induction of meiotic gynogenesis in bagrid catfish (*Pseudobagrus ussuriensis*) with homologous sperm and its confirmation for female homogamety

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Abstract

The bagrid catfish, *Pseudobagrus ussuriensis*, exhibits significant sexual dimorphism in growth rate and body size with males growing faster than females. Therefore, an all-male culture can dramatically increase the output and profitability of fishery products. According to the monosex breeding route, super-male individuals' acquirement by XY male sex reversal and artificial gynogenesis is the key step. An effective protocol to induce meiotic gynogenesis using homologous sperms has been developed in this study. The most effective UV irradiation for sperm genetic inactivation was found to be at a distance of 20 cm with 66 $\mu W/cm^2$ light intensity for 25 min. Optimal treatment for cold shock was 5 min post-fertilization at 0-4°C for 30 min, which gave the best survival rate of 13.65 $\pm$ 2.87%. The sex ratio in the meiotic gynogens showed a significant female-biased deviation ($p < .05$); thirty meiogynogens and their parents were further analysed using a male-specific AFLP marker, of which only the male parent produced a male-specific DNA band of 412 bp. These results indicated the female homogametic XX/XY sex determination system in *P. ussuriensis*. The optimization of a protocol for the successful induction of meiogynogenesis in the bagrid catfish lays the basis for all-male production and is useful in ascertaining the genetic sex determination system in this promising aquaculture species.

KEYWORDS
artificial gynogenesis, cold shock, *Pseudobagrus ussuriensis*, sex determination system, UV irradiation

1 | INTRODUCTION

Gynogenesis is a form of uniparental genome inheritance from female without genetic contribution of sperm. It can also be artificially induced by fertilizing eggs with genetically inactivated sperm, following diploid restoration by the suppression of second polar body extrusion or first cleavage, leading to meiotic or mitotic gynogenesis respectively (Gui & Zhou, 2010; Komen & Thorgaard, 2007). The techniques are relatively simple with two steps: generally, inactivation of spermatozoa by UV irradiation or chemical treatments and fertilized egg shock treatments (thermal or pressure) for diploidization of maternal chromosome set (Ghigliotti, Bolla, Duc, Ottesen & Babiak, 2011; Lebeda, Dzyuba, Rodina & Flajshans, 2013; Nowosad, Kucharczyk, Liszewski, Targońska & Kujawa, 2014; You, Yu, Tan & Tong, 2008). Artificial gynogenesis has been widely used in the study of fish genetics and breeding like for the production of monosex fish (Luo et al., 2011; Purdom, 1986), establishment of inbred lines (Li, Liang, Luo, Pan & Zou, 2015) and identification of the sex determination system (Campos-Ramos, Harvey, Mcandrew & Penman, 2003;

Chen et al., 2012; Fopp-Bayat, 2010; Hassanzadeh Saber & Hallajian, 2014; Li et al., 2015; Luo et al., 2011; Purdom, 1986).

In many species of cultured finfish, there is a significant sexual dimorphism in growth rate and body size; therefore, there is an increasing interest in generating monosex brood stocks for cost-efficient aquaculture (Liu et al., 2013; Mair, Abucay, Skibinski, Abella & Beardmore, 1997). The combination of artificial gynogenesis and sex reversal is an important approach to obtaining monosex stock in aquaculture. Using this strategy, monosex strains have been developed in many fish species, such as black carp (*Mylopharyngodon piceus*) (Rothbard et al., 1997), crucian carp (*Carassius cuvieri*) (Luo et al., 2011), half-smooth tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2012; Ji et al., 2010), yellow catfish (*Pelteobagrus fulvidraco*) (Liu et al., 2013), tilapia (*Oreochromis niloticus*) (Mair et al., 1997) and turbot (*Scophthalmus maximus*) (Meng et al., 2016) and so on.

In gonochoristic fishes, only a few have been proven to own morphological differentiated sex chromosomes, most only have regional differentiation within the gene sequences of two sexes. Therefore, sex identification through the cytogenetical method is rarely possible. By scoring sex ratios of successfully induced gynogens, sex determination modes of some fishes have been clarified: in Mozambique tilapia (*Oreochromis mossambicus*) (Campos-Ramos et al., 2003), stinging catfish (*Heteropneustes fossilis*) (Christopher, Murugesan & Sukumaran, 2010) and turbot (*Scophthalmus maximus*) (Piferrer et al., 2004), sex determination was proven as the XX/XY pattern, while in paddlefish (*Polyodon spathula*) (Shelton & Mims, 2012), half-smooth tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2012), large-scale loach (*Paramisgurnus dabryanus*) (You et al., 2008), ship sturgeon (*Acipenser nudiventris*) (Hassanzadeh Saber & Hallajian, 2014), Siberian sturgeon (*Acipenser baeri*) (Fopp-Bayat, 2010), rosy bitterling (*Rhodeus ocellatus ocellatus*) (Kawamura, 1998) and turbot (*Scophthalmus maximus*) (Meng et al., 2016),ZZ/ZW was believed to be the sex determination type.

Pseudobagrus ussuriensis, which is widely distributed in East Asia, is the largest of the bagrid catfish species (Pan, Li, Zhou, Qiang & Gui, 2015). With its chewy flesh and appealing taste, this fish is well liked by Chinese consumers (Wang et al., 2014). Sexual dimorphism on growth rate and body size is significant in *P. ussuriensis* with males being threefold larger than females in aquaculture. It means that an all-male culture can dramatically increase the aquaculture output and profitability of this fish (Gui & Zhu, 2012; Mei & Gui, 2015). The male heterogametic sex determination mode has been suggested in *P. ussuriensis* in our previous study (Pan et al., 2015). According to the monosex breeding route, acquirement of super-male individuals by XY male sex reversal and artificial gynogenesis is the key step. In this study, meiotic gynogenesis for *P. ussuriensis* was optimized through inactivation of spermatozoa using UV irradiation and diploidization of maternal chromosome set by cold shock. The sex determination system along with female homogamety of this bagrid catfish was confirmed by calculating the sex ratio of gynogens using an amplified-fragment length polymorphism (AFLP) marker.

2 | MATERIALS AND METHODS

2.1 | Broodfish and gamete collection

Matured broodfish were collected from Huaian Fisheries Research Institute (Jiangsu province, China) in June 2014 and temporarily reared in a cement pool with continuous aeration. Female parents were twice injected (with a 12-hr interval) using mixed spawning stimulation hormones (2,000 IU/kg HCG, 20 μg/kg LHRH-A), and males were injected only once with half the dose of the mixed hormones (Liu et al., 2013). After about 12 hr, gonads matured and females were covered by a piece of cotton cloth and gently massaged from the abdomen towards the anus to collect eggs into a plastic basin. As milt of artificially stimulated male *P. ussuriensis* cannot be extruded, males were dissected after anaesthetization by MS222, and milt was collected and preserved in Hank's balanced salt solution (diluted to 1:5), which was derived from testicular homogenate extracts. The sperm suspension was kept in a refrigerator at 4°C until it was used for UV inactivation.

2.2 | Optimization of genetic inactivation of the sperm

The sperm suspension was poured into a petri dish (9.0 cm diameter) to form a 1 mm depth layer and subjected to UV irradiation. Two 18-W (2537Å) germicidal lamps were fixed on top of the liquid surface at a distance of 20 cm, which provided a light intensity of about 66 $\mu W/cm^2$. Nine dishes of sperm suspensions were irradiated for 0, 5, 10, 15, 20, 25, 30, 35 and 40 min respectively, and then, sperm motilities for these treatment groups were microscopically assessed. After UV irradiation, sperm suspension of each treatment group was mixed with 30 ml of eggs and immediately activated with 15 ml of physiological saline for fish, after which the fertilized eggs were incubated in aerated circulating water at 25 ± 1°C. Each UV treatment experiment was replicated three times. The entire process was carried out in the dark to prevent genetic photoreactivation (Hassanzadeh Saber & Hallajian, 2014). The rates of fertilization, hatching and larvae surviving were respectively determined as in the previous description (Ji et al., 2010; You et al., 2008). A maximum UV irradiation dosage with high sperm motility alone was used for the next experiments (Christopher et al., 2010).

2.3 | Retention of the second polar body by cold shock

One batch of eggs was fertilized with non-irradiated sperm as the normal control, and another batch was fertilized with irradiated sperm as the haploid control. The remaining batches were activated with UV-irradiated sperm suspension (the irradiated spermatozoa that yielded 0% survival with a high motility alone were used for inseminating eggs) and then cold-shocked. According to our observation, the first cleavage of the fertilized eggs occurred at about 15 min post-fertilization (p.f.) at 26°C. Eggs activated with

UV-irradiated sperm were subjected to cold shock in a 0-4°C water bath at 5 min p.f., and the shock durations lasted for 5, 10, 15, 20, 25, 30, 35, 40 min, respectively, in order to suppress extrusion of second polar bodies. Each batch of fertilized eggs was treated as described above and hatched in different aquariums. All trials were performed three times using eggs derived from different females. Some of the developing eggs and embryos were observed under a stereo zoom microscope (Olympus SZX16) for percentage calculation.

2.4 | Sex examination and verification of meiotic gynogenesis with AFLP marker

Gynogenetic fry derived from different female fish were reared separately along with the control. After growing for 5 months, fish were randomly sampled for sex identification, which was based on morphological characters: males have projected anal papillae and females have an anal opening (Lim et al., 2013). Thirty randomly selected individuals from a putative meiotic family were sampled, and fin clips, including two parental individuals, were stored in absolute alcohol. Genomic DNA was isolated from fin clips using a DNA extraction kit (Clontech, NucleoSpin® Tissue) according to the manufacturer's instructions. AFLP analysis was conducted using a male-specific primer pair (F: 5′-TGT TGA GCG TGA TGT GAG TGA GC-3′, R: 5′-AAA CAA ACA CCA GGG CAG GAC TA-3′) as per previous description (Pan et al., 2015). The PCR products were separated using 6% denaturing polyacrylamide gel (Bio-Rad, Sequi-Gen GT System) and visualized via silver staining (Li et al., 2016; Wang, Mao, Chen, Liu & Gui, 2009).

2.5 | Statistical analysis

All data were standardized to the relative percentage to reduce the maternal effect among the experiments (Christopher et al., 2010) and expressed as the mean ± SD. Analysis of variance (ANOVA) was used to determine significant differences between groups by SPSS15.0. When differences were significant, the *t* test was used for comparison. Effects were considered as significant at $p < .05$. Comparisons of the sex ratio against the expected ratio (1:1) were performed using the chi-square test.

3 | RESULTS

3.1 | Determination of UV irradiation duration and haploid gynogenesis

The eggs fertilized with non-irradiated sperm produced the highest fertility and hatching percentage, reaching 86 ± 3.25% and 75 ± 0.78%. The fertilization rate and hatching rate decreased from 5 min to 15 min, but increased from 20 min to 25 min, reaching higher values of 29 ± 1.87% and 18.92 ± 2.39% at 25 min. Once again, the corresponding values showed a decreasing trending beyond 30 min of UV irradiation (Figure 1).

The majority of the eggs fertilized with UV-irradiated sperm for <15 min died during the embryonic development. Some of the eggs developed to the blastula stage and a few were able to hatch out, but some larvae displayed various deformities resulting in mortality. The hatched fry from the eggs inseminated with UV-irradiated sperm for ≥20 min all died before the first feeding. These fry showed a typical haploid syndrome characterized by an enlarged cardiac cavity and a distorted body (Christopher et al., 2010) (Figure 2). The UV-irradiated duration of 25 min was selected in the next trials for its higher fertility and hatching percentage.

3.2 | Optimization of cold shock treatment

The highest fertility, hatching and survival rates were observed in the normal control group. There was no significant differences in fertilization and hatchery rates between the haploid control group and experimental groups ($p > .05$), and there were no survivors in the haploid control group at the mouth-opening stage. For cold shock groups, the survival ratio increased along with lengthening of cold shock durations and reached the highest survival rate (13.65 ± 2.87) at a cold shock duration of 30 min, while after 30 min of cold shock, the survival rate decreased (Table 1).

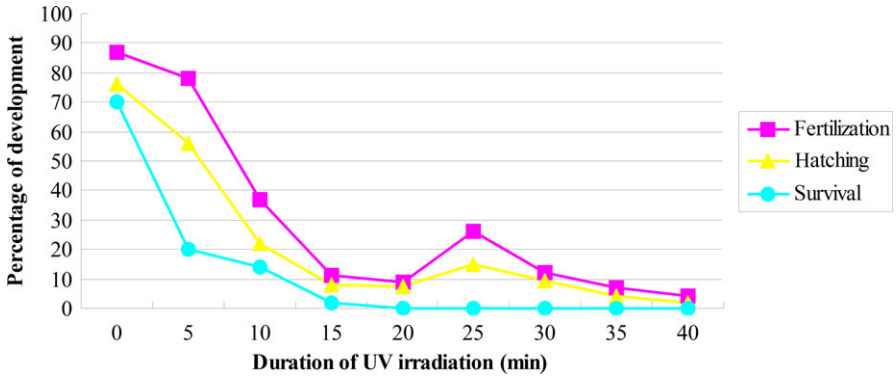

FIGURE 1 Development status of eggs inseminated with different UV irradiation sperm [Colour figure can be viewed at wileyonlinelibrary.com]

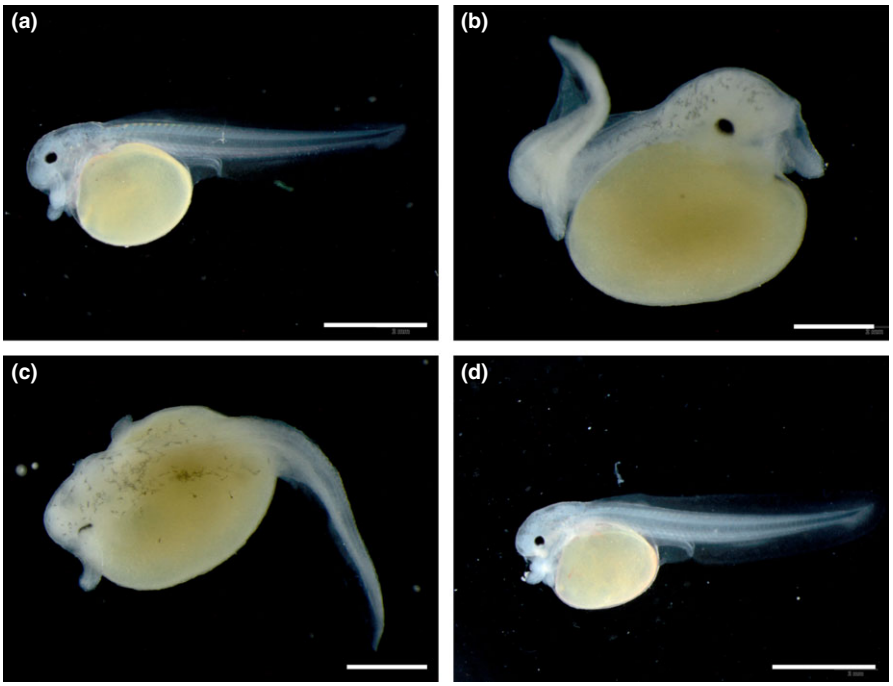

FIGURE 2 Morphology of normal and abnormal fry was shown just after hatching. The appearance of normal larva derived from the normal control group (a). The malformation of fry produced in haploid control group (b, c). The gynogenetic larva came from cold shock groups (d). In (a) and (d), scale bars=1 mm; in b and c, scale bars=500 μm [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Screening of the cold shock duration for retention of the second polar body

Group	Duration (min)	Fertilization rate (%)	Hatching rate (%)	Survival rate (%)
Normal control	0	86 ± 3.25	75 ± 0.78	70 ± 4.23
Haploid control	0	31 ± 3.12^{a}	21.20 ± 2.27^{a}	0
Cold shock 2	5	27 ± 1.21^{a}	21.71 ± 0.45^{a}	5.11 ± 1.56^{a}
Cold shock 3	10	32 ± 2.56^{a}	18.53 ± 5.12^{a}	8.28 ± 2.58^{a}
Cold shock 4	15	30 ± 3.18^{a}	19.14 ± 1.23^{a}	9.53 ± 2.21^{b}
Cold shock 5	20	28 ± 1.45^{a}	18.88 ± 1.07^{a}	10.82 ± 3.03^{b}
Cold shock 6	25	29 ± 1.87^{a}	18.92 ± 2.39^{a}	11.26 ± 0.89^{bc}
Cold shock 7	30	31 ± 2.35^{a}	20.24 ± 3.01^{a}	13.65 ± 2.87^{bc}
Cold shock 8	35	29 ± 4.12^{a}	22.17 ± 2.04^{a}	8.89 ± 3.56^{b}
Cold shock 9	40	30 ± 0.67^{a}	21.41 ± 1.09^{a}	4.37 ± 1.45^{a}

Values marked with different letters indicate significant difference ($p < .05$).

For larvae morphology, the majority of fry in the haploid control group showed a typical "haploid syndrome" such as a shortened body, kyphosis, bent tail and enlarged pericardium (Figure 2b,c), and none survived to the first feeding stage. In the normal control and cold shock groups, the fries displayed similar body lengths and appearances (Figure 2a, d), but lower rates of fertilization, hatching and survival were observed in cold shock groups compared with the normal control group.

3.3 | Sex ratios of gynogenetic progenies

After 5 months of rearing at room temperature 26 ± 1°C, the sex ratios in the gynogenetic progenies from three females, as well as the normal control group, were determined by detecting their phenotypic sex. The sex ratio in the meiotic gynogens showed a significant female-biased ($p < .05$) deviation compared to the 1:1 sex ratio in the normal control group ($p > .05$) (Table 2). These sex ratio data, to some extent, supported the putative XX/XY sex determination system in *P. ussuriensis*.

3.4 | Analysis of genetic characteristic using AFLP marker

Thirty meiogynogens and their parents were analysed using a male-specific AFLP marker, of which only the male parent produced a male-specific DNA band of 412 bp but the male-specific band was

TABLE 2 Sex ratio of normal control, gynogens of three female parents in *Pseudobagrus ussuriensis*

Group	Sample number	Males	Females	Female ratio (%)	χ^2(1:1)
Normal control	60	28	32	53.33	0.133[a]
Gynogen 1	26	1	25	96.15	11.077[b]
Gynogen 2	17	0	17	100	8.500[b]
Gynogen 3	24	2	22	91.66	8.333[b]

Values marked with different letters differ significantly ($p < .05$).

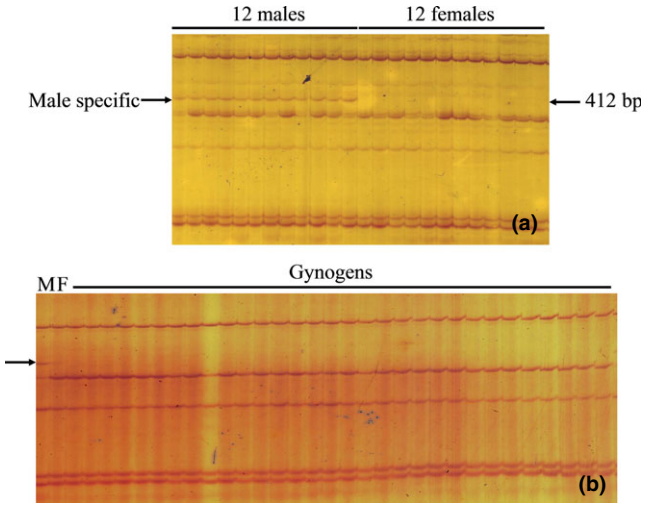

FIGURE 3 Genetic sex identification of *Pseudobagrus ussuriensis* using male-specific amplified-fragment length polymorphism marker. A male-specific DNA band of 412 bp in 12 males and 12 females (a). Genetic characteristic analysis in 30 meiogynogens and their parents, the male-specific band existed only in the male parent (b). M, male parent; F, female parent [Colour figure can be viewed at wileyonlinelibrary.com]

not detected in the female parent and the gynogens (Figure 3), indicating that no XY male was produced in the tested meiogynogens. This result further confirmed that the sex determination system could be the female homogametic XX/XY system in *P. ussuriensis*.

4 | DISCUSSION

Gynogenesis is an effective method for obtaining genetically inbred fish lines and exploring the sex determination system. In this study, a protocol to produce gynogenetic *P. ussuriensis* was developed, involving a combination of sperm inactivation of UV irradiation and a subsequent cold shock to retain the second polar body of fertilized eggs. No viable hatched fry could survive up to the first breeding when the eggs were fertilized with sperms irradiated for ≥20 min without cold shock, indicating that sperms were completely inactivated at this time. During 20-35 min irradiation, the fertilization and hatching rate first increased and then decreased. This paradoxical curve resulted in a typical Hertwig effect (Arias-Rodríguez, Rodríguez-Ibarra & Valle-Pignataro, 2004; Ijiri & Egami, 1980; Piferrer et al., 2004; Samonte-Padilla, Eizaguirre, Scharsack, Lenz & Milinski, 2011). The sperms that were irradiated by UV for 25 min had the highest fertilization ability; therefore, this irradiation duration is suitable for sperm inactivation in this species.

In most gynogenesis studies, both homologous and heterologous sperms were used to activate egg development (Adam Luckenbach et al., 2004; Dobosz et al., 2015; Meng et al., 2016). The two strategies both have advantages and disadvantages in the gynogenetic studies. The percentage of viable gynogenetic larvae induced by homologous sperm is apparently higher than that induced by heterologous sperm, but it is hard to exclude the incomplete inactivation of spermatozoa and gynogenetic diploids is not easy to distinguish from normal diploids when homogeneous sperm was used. The polymorphic molecular markers such as AFLP and SSR can help to solve these problems by identifying the maternal genetic characteristics (Dan, Mei, Wang & Gui, 2013; Zou, Wei & Pan, 2011). Utilization of heterologous sperm, especially with a different chromosome number, does not truly fertilize eggs for producing hybrids between distantly related species, ensuring that surviving larvae are indeed gynogens as the hybrids either showed a morphological marker or are absolutely lethal (Rothbard et al., 1997), but the fertilization rate and the diploid induction rate are lower than that induced by homologous sperm (Chen et al., 2012; Ji et al., 2010).

Cold shock is simple and easily conducted with no special apparatus necessary compared with pressure shock. It could be inferred that the diploidization of the maternal chromosome set by cold shock at 5 min p.f. was the result of second polar body retention because the first cleavage of the "fertilized" egg occurred at about 15 min p.f. The initiation time of the first mitotic cleavage blocking in most fishes is more than 20 min (Chen et al., 2012; Lin, Zhu, You, Wu & Cao, 2015). The untreated "fertilized" egg that developed into haploid syndrome's fry indicated that cold shock could induce the maternal chromosome set diploidization. The current obstacles of artificial gynogenesis in application are low yields and survival ratios. The highest survival rate in this study of 13.65% with 30-min shock duration could provide adequate breeding materials for future studies.

By measuring the sex ratio of gynogenetic diploids, the sex determination system can be determined (Campos-Ramos et al., 2003; Chen et al., 2012; Hassanzadeh Saber & Hallajian, 2014; Komen & Thorgaard, 2007). Therefore, gynogenesis is a useful technique for studies on sex determination mechanism in fish. In the present study, the sex ratios of gynogenetic progenies from three females were significantly female biased. Meanwhile, results of molecular analysis by the male-specific AFLP marker showed that no male-specific bands in gynogenetic progenies were detected. These results strongly proved that the sex determination system in *P. ussuriensis* is the female homogametic XX/XY system, which also further verified our previous inference (Pan et al., 2015). As for the emergence of very few male individuals in meiogynogens, possible reasons might be: (1) sex determination and differentiation in this species are influenced by environmental factors such as temperature,

pH, (2) autosome or polygenic loci participate in sex determination and differentiation in bagrid catfish and (3) spermatozoon is inactivated incompletely or implementation is contaminated by experimenter carelessness. It is necessary to investigate the major sex determination gene(s) of *P. ussuriensis* in future.

In consideration of food security, using the hormone-induced sex reversal fish as parents to breed monosexual offspring is a reliable method in aquaculture. Oestrogen-induced sex reversal and artificial gynogenesis can acquire YY super-males from the sex reversal progeny, and thereby produce all-XY males by the mating of YY super-males and XX females (Gui & Zhu, 2012; Mei & Gui, 2015). It will evidently increase the feed conversion ratio and output in all-male culture by the combination of sex reversal and artificial gynogenesis in *P. ussuriensis*.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interests.

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