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Production of functionally sterile triploid Nibe croaker *Nibea mitsukurii* induced by cold-shock treatment with special emphasis on triploid aptitude as surrogate broodstock

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ABSTRACT

Among the pelagic egg spawning marine teleosts, spermatogonial transplantation techniques were first developed in the Nibe croaker *Nibea mitsukurii*; however, production of donor-derived offspring had not yet been achieved. In salmonids, when triploid individuals were used as recipients, the resulting surrogate broodstock produced only donor-derived gametes, as the triploids were incapable of producing their own gametes. Therefore, in the present study, we determined the optimal parameters for cold-shock inducing triploidy through the suppression of meiosis II resulting in retention of the second polar body in fertilized Nibe croaker eggs. It was determined that cold-shock treatment, which was carried out at 10 °C for a period of 15 min beginning 5 min after fertilization, following pre-shock treatment at 22 °C achieved a 100% triploidization rate as well as hatching rates of which were high enough for practical application in the pelagic egg spawning marine teleost (16.4 ± 10.4%). Average chromosome numbers of cold-shock treated larvae counted using mitotic figures found on the fin membrane of 1 day post-hatch larvae were 67.3 ± 0.7, which was approximately 1.5 times larger than that of control larvae (46.8 ± 1.1), suggesting that triploidy had been induced. No differences in survival rates of hatched larvae during the rotifer-feeding stage existed between treated and non-treated groups. Gonadosomatic index and gametogenesis were impaired in both sexes of triploid Nibe croaker. Although triploids produced small amounts of gametes, no progeny from crosses between triploids and diploids survived later than 24 h subsequent to hatching. These results indicated that the triploid Nibe croaker developed in this study could be effectively used as surrogate recipients in spermatogonial transplantation.

Statement of relevance: In intraperitoneal germ-cell transplantation techniques in salmonids, donor-derived diploid germ cells proliferated and differentiated within triploid recipient gonads, and the surrogate recipients produced only donor-derived gametes, which were capable of generating viable offspring when used in fertilization. To date, germ-cell transplantation techniques have been developed for use in perciforms, including sciaenids, however, triploid recipients have not yet been utilized in marine fishes. In this study, triploid Nibe croaker was produced at high triploidization rates (~100%) using cold-shock treatments. Analysis of the gonadal development and gametogenesis of triploid Nibe croaker in two consecutive spawning seasons revealed that the triploid Nibe croaker was functionally sterile in both sexes and that the triploids would be suitable for use as surrogate recipients.

1. Introduction

Physiological changes that occur in accordance with the sexual maturation of fish species targeted for use in aquaculture, such as decreases in both body growth rates and disease tolerance, result in increased feeding costs and mortality rates, and price decline due to inferior flesh quality of aquaculture stock (Qin et al., 1998; Piferrer et al., 2009). To avoid these problems, triploidization techniques have been developed in order to produce functionally sterile fishes for use in aquaculture. In some triploid fishes, it has been reported that meiosis is seriously affected, as three homologous chromosomes cannot correctly pair during the zygotene stage of prophase I (Benfey, 1999; Carrasco et al., 1998; Cuñado et al., 2002; Myers, 1986). In fact, reduced gonadal development and abnormal gametogenesis have been observed in almost all triploid fishes examined (Piferrer et al., 2009).

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Reproductive abnormalities resulting from triploidy have significant applications with respect to aquaculture and aquaculture biotechnology. Induced triploidy has been exploited for the genetic containment of farmed and genetically modified fishes, as sterility can prevent genetic contamination with local gene pools and propagation of exotic species in the natural environment resulting from escapees (Piferrer et al., 2009). The authors of the present study recently developed a germ cell transplantation technique for use in salmonids that employs triploid and diploid fish as the recipient and the donor, respectively (Okutsu et al., 2006, 2007, 2008; Yoshizaki et al., 2010, 2011). In these experiments, progenitor cells of gametes, such as type A spermatogonia and oogonia, from diploid (2n) donor fish were microinjected into the peritoneal cavities of newly hatched triploid (3n) larvae. After transplantation, some donor germ cells actively migrated toward, and subsequently colonized, recipient genital ridges. Donor-derived diploid germ cells proliferated and differentiated within triploid recipient gonads, and recipients produced only donor-derived gametes, which were capable of generating viable offspring when used in fertilization (Okutsu et al., 2007, 2008). This fact suggested that the triploid recipients possess abilities to nurse diploid germ cells till they mature. Indeed, although meiosis is usually impaired in the germ cells of triploids, the triploid gonad does possess the ability to synthesize sex steroids required for gametogenesis (Felip et al., 2001a). To date, germ cell transplantation techniques have been developed for use in perciforms, Nibe croaker *Nibea mitsukurii* (Takeuchi et al., 2009; Higuchi et al., 2011), chub mackerel *Scomber japonicus* (Yazawa et al., 2010), and yellowtail *Seriola quinqueradiata* (Morita et al., 2012, 2015); however, the production of donor-derived offspring using diploid recipients was only successfully reported in allogenic and xenogenic germ cell transplantation using yellowtail donor (Morita et al., 2012, 2015). Furthermore, germ cell transplantation using triploid recipients has not yet been utilized in marine fishes. In addition to efficient donor-derived gametogenesis, the authors of the present study found that using triploid recipient salmonids allowed for the restoration of fertility in triploid recipient fish, and that the fecundity of those fish was quite similar to that of a control diploid group (Yoshizaki et al., 2011). In the present study, the authors sought to achieve functional sterilization by induction of triploidy in a marine fish recipient, Nibe croaker, for use in germ cell transplantation.

Nibe croaker is distributed throughout the sea of Japan and the East China Sea (Kinoshita and Fujita, 1988) and is a commercially valuable sciaenid fish. The species was one of the first marine species selected for use in germ-cell transplantation technique (Takeuchi et al., 2009; Higuchi et al., 2011), because it possesses a short generation time (4 to 6 months to reach sexual maturity), is capable of spawning multiple times in a controlled environment, and its broodstock and larvae are easy to maintain in small aquarium tanks. Nibe croaker was shown to have a chromosome number of 2n = 48 (Ojima and Kikuno, 1987). The induction of triploidy has been achieved in many fish species using treatments designed to prevent extrusion of the second polar body of fertilized eggs (Maxime, 2008). Among the sciaenids, induction of triploidy was achieved in shi drum *Umbrina cirrosa* (Libertini et al., 1999; Ballarin et al., 2004; Segato et al., 2006) and large yellow croaker *Pseudosciaena crocea* (Lin et al., 2001; Wang et al., 2001; Xu et al., 2006, 2008). In both species, triploids were obtained using cold-shock treatments, with shocks carried out at temperatures of 2 to 8 °C for periods of 8 to 14 min beginning 1.5 to 5 min after artificial insemination (Ballarin et al., 2004; Segato et al., 2006; Wang et al., 2001; Xu et al., 2006). The major variables influencing the effectiveness of temperature shock were timing, intensity, and duration of shocks. The critical values for each variable were species-specific, and optimization of all three factors was required in order to obtain the highest triploid yield. In the present study, the authors aimed to develop a triploidization protocol using cold-shock treatments for use in Nibe croaker by preventing extrusion of the second polar body. As the extent of reduction and abnormality of reproductive performance of triploid fish are highly variable between both species and sexes (Felip et al., 2001b), the authors also examined the gonadal development of triploids for 21 months, a period that encompassed two full consecutive reproductive cycles, and the fertility of resulting gametes.

2. Materials and methods

2.1. Broodstock management, gamete collection, and artificial insemination

All experiments were conducted at Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (Chiba, Japan) in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology. Two years old Nibe croaker broodstock (n = 20) whose total length was ranged from 15 to 20 cm were maintained in circular Fiber Reinforced Plastic (FRP) tanks (1000 l). In order to facilitate year-round spawning, water temperature was maintained between 22–26 °C using aquarium heaters and photoperiod was fixed at 13 h light (from 06:00 to 19:00). Under these conditions, spawning of Nibe croaker occurred between 20:00 to 23:00. Of 20 females reared in the broodstock tank, one or two ovulating females spawned each day. After 20:00, females were anesthetized one time per hour and their abdomens were gently squeezed by hand in order to obtain eggs immediately after ovulation for use in artificial insemination. Ovulated eggs from these female specimens were then collected into a 1000-ml glass bowl. 1.5 ml of milt was collected from each of 2 to 4 similarly anesthetized males by pressing their abdomens, diluted 10 times with balanced salt solution for marine teleosts (13.5 g/l NaCl, 0.6 g/l KCl, 0.25 g/l $CaCl_2$, 0.2 g/l $NaHCO_3$, 0.35 g/l $MgCl_2$) used as an artificial seminal plasma, and stored on ice prior to use. One batch of eggs (approximately 50,000) was gently mixed with 15 ml of diluted milt, and sperm was subsequently activated by adding 500 ml of 22 °C seawater. Fertilized eggs were rinsed twice with 500 ml of 22 °C seawater and stored in the incubator at 22 °C prior to cold-shock treatment.

2.2. Experiment design for cold-shock treatments

Three treatment variables affecting production of triploid larvae were studied: 1) cold-shock temperature, 2) cold-shock duration, and 3) the optimal time for initiation of cold-shock treatment after fertilization. In experiment 1 (Exp-1), optimal cold-shock temperature and duration with respect to production efficiency (%) of triploid larvae (hatching rates × triploidization rates) were determined. Cold-shocks commenced at 3 min post-fertilization (mpf), and were applied for durations of either 15, 30, 45, or 60 min at either 5, 10, or 15 °C. In experiment 2 (Exp-2), shocks commenced at 3 mpf, and were applied for durations of either 5, 10, 15, 20, 25, or 30 min at either 5 or 10 °C. In experiment 3 (Exp-3), in order to determine the optimal time for initiation of cold-shock treatment after fertilization with respect to production efficiency of triploid larvae, 15-min cold shocks at 10 °C were initiated at 1, 3, 5, 7, 10, and 15 mpf. Each treatment group consisted of approximately 2000 fertilized eggs. Non-treated control groups consisted of gamete samples obtained from the same Nibe croaker specimens. Each cold-shock treatment was repeated for three times using different batches of fertilized eggs.

2.3. Fertilization, normal cleavage, hatching, and survival rates of cold-shock treated embryos

Subsequent to cold-shock treatments, eggs were reared at 22 °C in 1000-ml glass beakers. One hundred floating eggs at 2 h post-fertilization (hpf) were collected on a plastic petri dish and observed under a stereo microscope (SZX-12, Olympus) and the number of fertilized embryos and the number of embryos were examined and represented as fertilization rates. At 2 hpf, embryos were developed to 32- to 64-cell stages, therefore the number of embryos exhibited normal and abnormal cleavage were counted and normal cleavage rates were

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