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The Use of Ultrasound to Enhance-transport of Compounds into Fish and Fish Embryos: A Review

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Abstract

Fish culture is a rapidly growing industry. Even faster growth can be expected if we have better control over reproduction, seed storage, growth, and diseases. Despite an availability of a variety of substances with the potential to allow control over processes that facilitate sexual differentiation, preservation of embryos, reproduction, and disease prevention, there is no reliable and efficient method to deliver these substances. Novel techniques such as ultrasound have shown to enhance transport of substances through the skin of both, mammals and fish. This paper summarizes results of some of our original studies to deliver calcein into fish larvae for marking and quantification, to enhance delivery of androgen for sex reversal of tilapia and to enhance permeation of cryoprotectants into embryos for cryopreservation using cavitation level ultrasound.

Introduction

Aquaculture is one of the fastest growing agricultural industries (17%) estimated to produce 38 to 40 million MT of fish annually (FAO 1999; De Silva 2000). Even with most conservative estimates, global demand for aquaculture product is expected to reach 183 million MT (increase of 110%) by 2030 (Ye 1999). As quality and quantity of land and water resources become increasingly scarce due to competing uses, aquaculture must move towards intensification. Intensification of aquaculture also requires that we have better control over culture animal's life cycle and it's environment. Control over reproduction, seed storage, growth, and disease would significantly add to the advancement of aquaculture and take us closer to meeting the projected 183 million MT goal by year 2030.

A variety of substances are available today with the potential to control processes that facilitate sexual differentiation, cryopreservation of gametes and embryos, reproduction, growth, and disease prevention and treatment.

An appropriate and efficient method to deliver these substances into fish, eggs and/or embryos is largely unavailable. Unfortunately, the conventional methods of delivery such as immersion, oral administration, power spray and injection are either inefficient or ineffective. Directly injecting the compound of interest into fish represents the most effective means of delivery (Palm et al. 1994). However, the disadvantages of this method include having to handle individual fish, making it labor intensive, stressful to fish and difficult to treat large populations. Treatment of fish during early developmental stages is particularly difficult when mortality associated with factors such as diseases and handling is high. A nonlethal and nonstressful means of delivering a controlled dose of compound to a large number of fish is needed. A more novel technique such as ultrasound has shown to enhance transport of substances through the skin of both, mammals (insulin, interferon) (Mitrogotri et al. 1995a) and fish (Calcein, androgens, GnRH and gentamycin) (Zohar et al. 1991, Bart et al. 2001).

Ultrasound effects on fish

Ultrasound has been used to help shatter kidney stones and accelerate chemical reactions (Machanshetty 1995, Prospetti 1986, Apfel 1981). Acoustic cavitation thought to enhance diffusion of substances into cells involves a sequence of events including nucleation, bubble formation, oscillation and implosive collapse of air bubbles. It results from an ultrasound wave that produces alternating regions of compression and rarefaction, and consequently, pressure changes in the fluid (Williams 1983). Recent studies in human skin demonstrated that these oscillations, and the collapse of bubbles, effectively disorganize the lipid bilayers allowing larger protein molecules to diffuse through the human skin (Mitragotri et al. 1995a,b).

The teleost epidermis is composed of a stratified squamous epithilium that actively transports chemicals across this barrier. Since fish skin and gills are well adapted to exchanging molecules with the external environment, cavitation enhanced diffusion is believed more effective with fish. Frankel et al. (1999) used therapeutic high frequency level ultrasound (1 MHz and 2.2W·cm^2) and showed that while ultrasound transports some compounds across fish skin it does so by altering external epithelia. Two-hour post-treatment examination of skin tissue indicated that altered area was restored and no sign of infection that are normally associated with skin injury was observed. In this same study Frankel et al. (1999) demonstrated the transport of two types of particles, silver chloride (AgCl) tracers and bacterins from aquatic medium into goldfish skin using therapeutic level ultrasound at different sonication frequencies, intensities and durations. These findings and those reported by Zohar et al. (1991) indicate that this method also does not cause irreversible damage to fish health or wellbeing, and thus represents a potentially viable alternative to traditional methods of delivery.

Similar to these previous studies we attempted to enhance transport of calcein, a calcium binding fluorescent compound into rainbow trout,

Oncorhynchus mykiss, larvae using cavitation level ultrasound. We also attempted to understand whether the use of ultrasound is an appropriate method to increase the permeation of cryoprotectants using calcein (622 MW) as model for commonly used cryopretectant, methanol (32 MW) into developing embryos. Furthermore, we used ultrasound to enhance delivery of androgens to sex reverse tilapia using immersion treatment. This report reviews the results from some of these three studies.

The use of ultrasound to enhance transport of calcein into rainbow trout, Oncorhynchus mykiss, larvae

CALCEIN AS A MARKER AND A TEST COMPOUND

Enhanced diffusion of calcein into rainbow trout, *Oncorhynchus mykiss*, larvae was examined using cavitation level ultrasound. Calcein (2,4-bis-[N,N'-di (carbomethyl) -amino-methy] fluorescein) is known to chemically bind with calcium resulting in a marked increase in its fluorescence when complexed with alkaline earth metals (Wallach et al. 1959). This compound has been used to mark a number of fish species in immersion treatments with various concentrations (Mohler 1997, Monaghan 1993, Brooks et al. 1994). Immersion treatments of 2 to 28 hours were required for sufficient levels of calcein to diffuse with mortality exceeding 41% in some cases. Mohler (1997) conducted only subjective assessment of markings. Short duration and high concentration immersion treatment combined with enhanced transport using cavitation level ultrasound was expected to result in

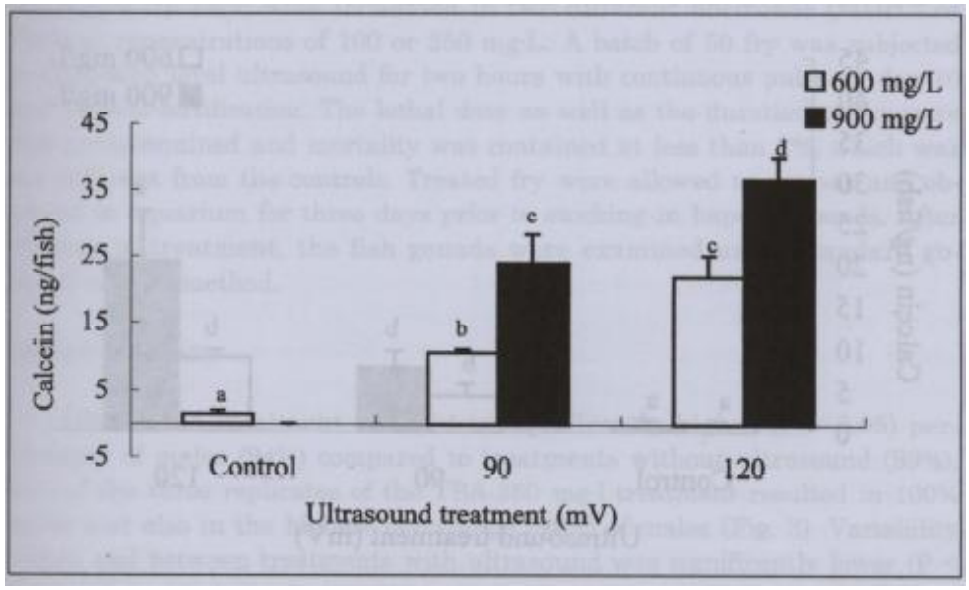

Fig. 1. Calcein in rainbow trout fry (n = 120) treated with 600 and 900 mg·l calcein, 90 and 120 mV (40 kHz) of ultrasound for 5 min. While there was no significant difference between those treated with two concentrations of calcein, a difference was found when larvae were treated with ultrasound. Bars with different letter superscripts indicate mean values that are significantly different ($P < 0.05$) (Bart et al. 2001).

accelerated rate of transport in our study. The objective of this study was to quantitatively evaluate the level of calcein transport and retention for 21 days post-treatment using cavitation level ultrasound for duration of 5 or 15 minutes.

Three variables consisting of two voltage amplitudes (90 mV and 120 mV), two calcein concentrations (600 and 900 mg·l) and two durations of treatments (5 and 15 min) were examined. Thirty-day old yolk sac fry (n = 640) were treated with calcein and/or ultrasound. After 21 days, treated larvae were individually homogenized, calcein extracted, filled in 96-well plate and emission read using cytofluorometer.

Overall mean diffusion of calcein ranged from 3.8 to 36.2 ng·fish (Figs. 1 and 2). The highest absorption level was observed with highest amplitude applied for 15 min in 900 mg·l of calcein solution (Fig. 2). Significantly higher diffusion resulted when treated with 120 mV compared against 90 mV ($P < 0.05$). Results from these experiments demonstrate that a several fold increase in the rate of diffusion of calcein into fish can be achieved when treated with cavitation level, low frequency ultrasound.

The use of ultrasound in improved sex reversal of Nile tilapia, Oreochromis niloticus

SUMMARY OF BACKGROUND AND METHODS

Tilapia nilotica, *O. niloticus*, was selected for this study because it is one of the most commonly cultured species. Tilapia is a prolific breeder and males usually grow faster than females. Propagation of all male population

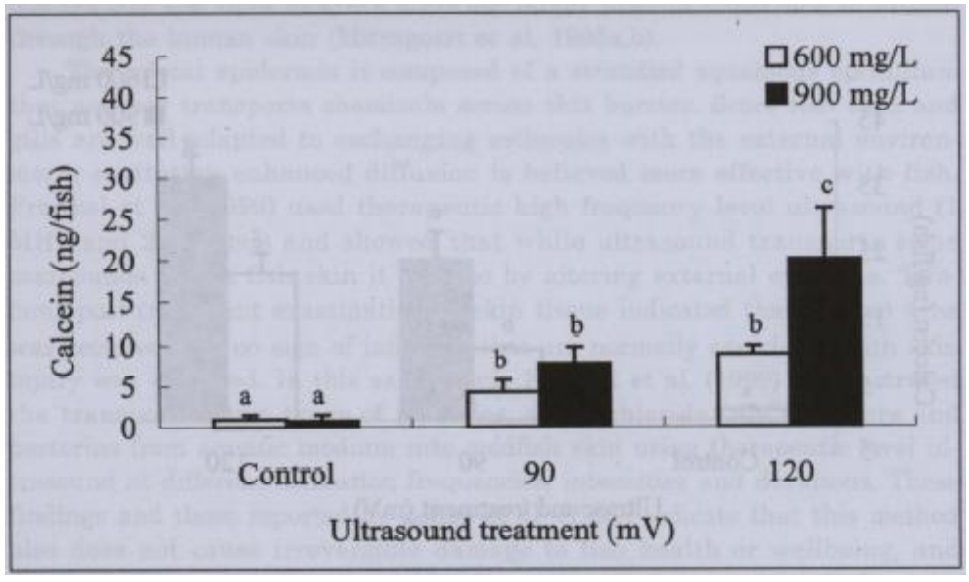

Fig. 2. Calcein in rainbowtrout fry (n = 120) treated with 600 and 900 mg·l calcein, 90 and 120 mV (40 kHz) of ultrasound for 15 min. Significant differences were found between those treated with two concentrations of calcein, and when treated with 0, 90 and 120 mV ultrasound. Bars with different letter superscripts indicate mean values that are significantly different ($P < 0.05$) (Bart et al. 2001).

is desirable to control reproduction and increase production. Methods to masculinize population have been developed using various kinds of androgens (see review, Pandian and Sheela 1995, Vardaraj, K. and T.J.Pandian 1987). A number of hormones have been found to be effective, although only 17α-methyltestosterone is used for commercial production. Current methods to masculanize population involve administration of androgen by incorporating in feed. The use of this method results in contamination of the environment (Fitzpatrick and Schreck 1999b). It is also inefficient because there is a rather uneven distribution of hormone as a consequence of feeding hierarchies. Additional loss of hormone results from uneaten food and dissipation in the water column. Immersion of fish in hormone containing solution has been used to masculanize salmonids and tilapia (Piffer and Donladson 1989, Fiest et al. 1995 and Fitzpatrick et al. 1998).

In a recent study Fitzpatrick and Schereck (1999a) achieved masculinization of 10-day old tilapia fry (20 to 92%) using methyldihydrotestosterone (MDHT), trenbolone acetate (TBA) and 17α-methyltestosterone (MT). Fish immersed in MT solution for 2 and 48 hours resulted in only 20 to 25% more males. This ineffective and inconsistent inversion of sex may be due to insufficient and non-uniform diffusion of hormone into tilapia larvae.

An immersion protocol combined with ultrasound exposure was expected to increase transport of hormones resulting in consistent masculinization of tilapia fry. The objective of our study was to assess the effect of cavitation level ultrasound on sex inversion using immersion protocol. Variables tested include two hormones (TBA and MDHT) at two concentrations (100 and 250 mg·l) and ultrasound (cavitation level).

In this study thirty separate experimental units containing batches of 50 tilapia fry each were immersed in two different hormones (MDHT or TBA) at concentrations of 100 or 250 mg·L. A batch of 50 fry was subjected to cavitation level ultrasound for two hours with continuous pulse on day 10 and 13 post-fertilization. The lethal dose as well as the duration of exposure was predetermined and mortality was contained at less than 2%, which was not different from the controls. Treated fry were allowed to recover and observed in aquarium for three days prior to stocking in hapas in ponds. After 60 days of treatment, the fish gonads were examined using standard gonadal squash method.

RESULT SUMMARY

Ultrasound treatment resulted in significantly higher ($P < 0.05$) percentages of males (94%) compared to treatments without ultrasound (89%). Two of the three replicates of the TBA-250 mg·l treatment resulted in 100% males and also in the highest percentage (98%) of males (Fig. 3). Variability within and between treatments with ultrasound was significantly lower ($P < 0.05$) (91 to 98%) than treatments with no ultrasound (83 to 94%). While there was no concentration effect, fry treated in TBA-250 mg·l and ultrasound resulted in significantly higher percentages of males (98.5%) than those treated with MDHT and ultrasound (90.5%). This study thus,

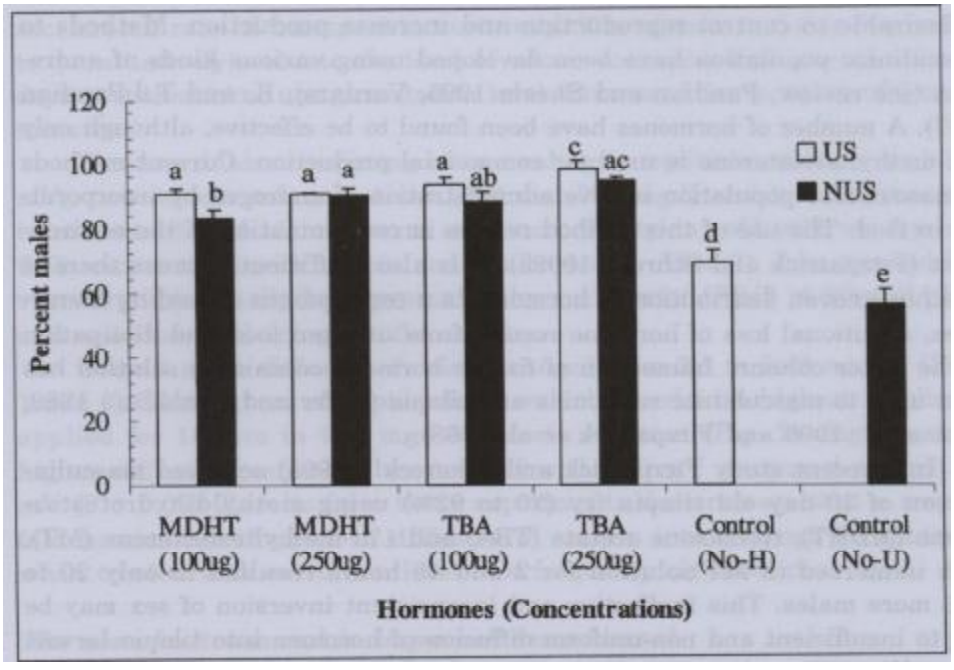

Fig. 3. Per cent males N. letilapia, O. N. loticus fry immersed in 17 α-MDTH or TBA at 100 or 250 ug·l with or without ultrasound (420 V and 47 KHz). The first control was treated with ultrasound without hormone and the second control was immersed in 5% ethanol solution without either ultrasound or hormone treatment. Significant differences were found between treatments with and without ultrasound as well as TBA and MDHT. Bars with different letter superscripts indicate mean values that are significantly different ($P < 0.05$) (Bart et al., in Press).

demonstrated the potential of a short-term immersion protocol using ultrasound to more predictably produce all-male, commercially viable, tilapia seed.

The use of ultrasound to enhance permeation of cryoprotectants into fish embryos

SUMMARY OF BACKGROUND AND METHODS

One of the primary obstacles facing aquaculture today is the lack of sufficient quality, quantity and year-round availability of fish seed. Inadequate supply is in part due to the necessity to store and transport fish seed similar to that of crops and livestock. Successful cryopreservation of fish gametes and embryos using relatively simple methods would provide a means of meeting these needs.

While we have successfully cryopreserved sperm from over 200 species, cryopreservation of eggs and embryos have not been successful in teleost species (Bart 2000). Difficulty in long-term preservation of embryos lies in our inability to introduce cryoprotectants at sufficient quantity and diffuse uniformly across embryos. Fish embryos are encased in multi-layer barriers with differing permeability to cryoprotectants (Hagedorn and Kleinhaus 2000). Understanding the kinetics of this phenomena and interactions with

embryo's biology is essential to developing a successful cryopreservation methodology. The purpose of this series of studies was to understand the embryo's ability to withstand exposure to commonly used cryoprotectants and to manipulate the rate of transport using cavitation level ultrasound.

Zebrafish embryos at two-cell, 32-cell, gastrula and epiboly stage (60 to 90%) were screened for cryoprotectant (DMSO, methanol, ethylene glycol and glycerol) tolerance at concentrations of 5,10, 20, 40, 50 and 60% (v:v). Furthermore, similar to the previous studies, these embryos were subjected to ultrasound in the presence of calcein to assess the effect on the permeability.

RESULTS SUMMARY

No viability was observed among embryos treated before epiboly stage of development. Lowest epiboly stage (10%) was viable when treated with lower concentrations of methanol. The overall results indicated that, 1) embryos at 90% epiboly or post-epiboly stage were more resistant to cryoprotectant toxicity; 2) methanol permeated the embryos and resulted in less mortality than other cryoprotectants tested; 3) longer exposure time (15 to 20 min) resulted in higher mortality; 4) methanol treated embryos survived as long as 20-minutes of immersion (Bart 1998).

With these findings, a second series of experiments were carried out to assess the impact of cavitation level ultrasound on embryos, embryos immersed in calcein solution and embryos immersed in methanol solution at

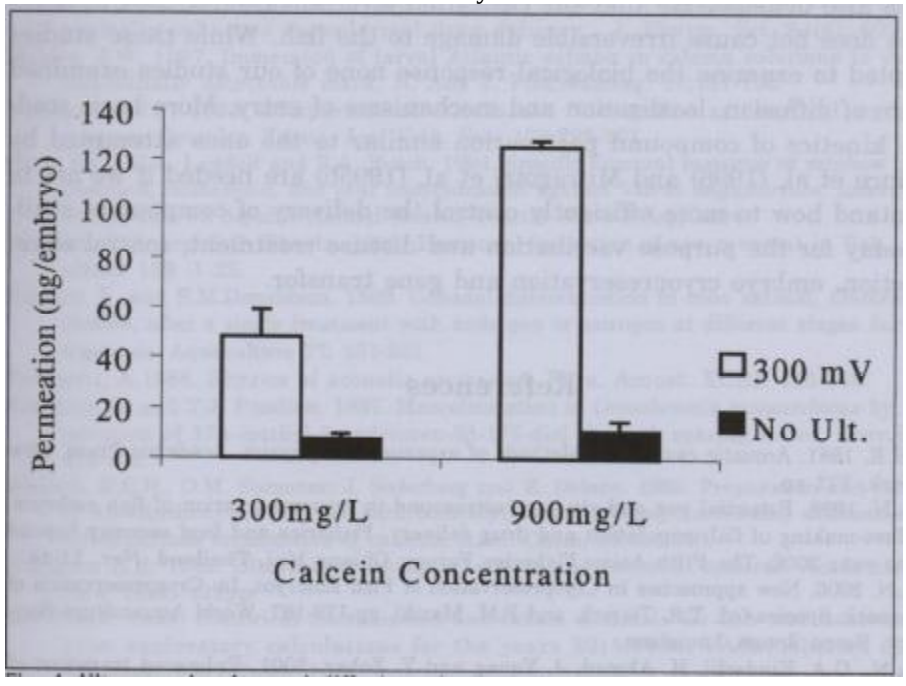

Fig. 4. Ultrasound enhanced diffusion of calcein into zebrafish embryo at epiboly (90%) stage of development. The embryos were treated with 300mV of ultrasound in two different concentrations (300 and 900 mg·L) of calcein for 10 min. Significant difference was found between two different concentrations. Control (no ultrasound exposure) had significantly lower amount of calcein compared with the treatments ($P < 0.05$) (Bart 1998).

various concentrations. Embryos suspended in calcein and treated with ultrasound for 10 min resulted in calcein level 4 to 5 times greater than without ultrasound (Fig. 4). Extraction protocols for this study was similar to the one described in the previous study on rainbow trout larvae (Bart et al. 2001). Higher concentrations also had significantly greater diffusion ratios.

A preliminary study assessed the voltage level to drive the cylindrical ceramic piezoelectric crystal transducer in order to induce cavitational ultrasound at which no mortality resulted. At this level we subjected embryos to concentrations of 5 to 60% of methanol. These studies revealed that the embryos consistently had higher survival (> 60%) when treated with as high as 45% methanol and as long as 3 to 4 min of ultrasound exposure.

This and previous studies strongly indicate the potential use of ultrasound in facilitating development of a successful cryopreservation protocol. A further study is underway to develop vitrification solution with 45% methanol and to carryout freezing trials at various temperature regimes.

Conclusion

These studies presented above and the previous studies indicate that ultrasound at both therapeutic levels and/or at low frequency cavitation level has the ability to enhance transport of compounds across fish skin and gills as well as across multiple compartment barrier of a developing embryo. Studies also demonstrate that the cavitation level ultrasound used in these studies does not cause irreversible damage to the fish. While these studies attempted to examine the biological response none of our studies examined the rate of diffusion, localization and mechanisms of entry. More basic studies on kinetics of compound permeation similar to the ones attempted by Hagedorn et al. (1996) and Mitragotri et al. (1995b) are needed if we are to understand how to more efficiently control the delivery of compounds available today for the purpose vaccination and disease treatment, control of reproduction, embryo cryopreservation and gene transfer.

References

Apfel, R.E. 1981. Acoustic cavitation. Methods of experimental physics. Academic Press, New York. 572 pp.

Bart, A.N. 1998. Potential use and study of ultrasound in cryopreservation of fish embryos, Mass-making of fish population and drug delivery. Fisheries and food security beyond the year 2000, The Fifth Asian Fisheries Forum: Chiang Mai, Thailand. Nov. 11-14.

Bart, A.N. 2000. New approaches in Cryopreservation of Fish Embryos. In: Cryopreservation of Aquatic Species (ed. T.R. Tiersch, and P.M. Mazik). pp.179-187. World Aquaculture Society, Baton Rouge, Louisiana.

Bart, A.N., G.A. Kindschi, H. Ahmed, J. Young and Y. Zohar. 2001. Enhanced transport of calcein into rainbow trout, *Oncorhynchus mykiss*, larvae using cavitation level ultrasound. Aquaculture 196:189-197.

Bart, A.N., A.R.S. Athauda, M.S. Fitzpatrick and W.M. Contreras-Sanchez. Ultrasound enhanced masculinizationof Nile tilapia, *Oreochromis niloticus* in immersion protocol. In Press.

Brooks, R.C., R.C. Heidinger and K.C. Christopher. 1994. Mass-marking otoliths in larval juvenile walleys by immersion in oxytetracycline, calcein and calcein blue. N. Am. J. Fish. Manag. 14:143-150.

FAO, 1999. Aquaculture Production Statistics. 1988-1997. FAO, Rome, Italy.

De Silva, S.S. 2000. A global perspective of aquaculture in the new millennium. In: International Conference on Aquaculture in the Third Millennium- Book of Synopses. Pp.51-100. Bangkok, Thailand.20-25 Feb. 2000.

Fiest, G., C.G. Yeoh, M.S. Fitzpatrick and C.B. Schreck. 1995. The production of functional sex-reversed male rainbow trout with 17a-methyl testosterone and 11b-hydroxyandrostenedione. Aquaculture 131: 145-152.

Fitzpatrick, M.S., W.M. Contreras-Sanchez, R.H. Milston, M. Lucero, G.W. Feist and C.B. Schreck. 1998. Steroid immersion for masculinization of Tilapia: Immersion of Tilapia fry I MDHT. PD/A CRSP, Sixteenth Annual Technical Report: 73-74.

Fitzpatrick, M. and C. Schreck. 1999a. Masculinization of Tilapia by immersion in Trenbolone acetate. PD/A CRSP, Ninth Work Plan: 10-13.

Fitzpatrick, M. and C. Schreck. 1999b. Fate of Methyltestosterone in the pond environment. PD/A CRSP, Ninth Work Plan: 26-29.

Frankel, V., E. Kimmel and Y. Iger. 1999. Ultrasound-induced cavitation damage to external epithelia of fish skin. Ult. Med. Biol. 25 (8): 1295-1303.

Hagedorn, M., E. Hsu, U. Pliatus, D.E. Wildt, W.F. Rall and S.J. Blackband. 1996. Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmantal biological system. Proceedings of the national academy of sciences 93:7454-7459.

Hagedorn, M. and F.W. Kleinhans. 2000. Problems and prospects in cryopreservation of fish embryos. In: Cryopreservation of Aquatic Species. (ed. T.R. Tiersch, and P.M. Mazik). World Aquaculture Society, Baton Rouge, Louisiana. Pp.161-179.

Machanschetty, S.I. 1995. Conceptual model for acoustic microcavitation. J. Acoust. Soc. Am. 98: 2681-2689.

Mitragotri, S., D. Blankschtein and R. Langer. 1995a. Ultrasound-mediated transdermal protein delivery. Science 269: 850-853.

Mitragotri, S., D. Blankschtein and R. Langer. 1995b. A mechanistic study of ultrasonically-enhanced transdermal drug delivery. J. Pharm. Sci. 84(6): 697-706.

Mohler, J.W. 1997. Immersion of larval Atlantic salmon in calcein solutions to induce a non-lethally detectable mark. N. Am. J. Fish. Manag. 17:751-756.

Monaghan, J.P. 1993. Comparison of calcein and tetracycline as chemical markers in summer flounder. Trans. Am. Fish. Soc. 122:298-301.

Palm, R.C., M.L. Landolt and R.A. Busch. 1994. Specific humoral response of rainbow trout to injection, immersion, and oral immunization against *Vibrio angullarium*. International Symposium on Aquatic Animal Health, Seattle WA 4-8 Sep, 1994.

Pandian, T.J. and S.G. Sheela. 1995. Hormonal induction of sex reversal in fish. Aquaculture 138 :1-22.

Piferrer, F. and E.M.Donaldson. 1989. Gonadal differentiation in coho salmon, *Oncorhynchus kisutch*, after a single treatment with androgen or estrogen at different stages during ontogenesis. Aquaculture 77: 251-262.

Prospetti, A.1986. Physics of acoustic cavitation. Phys. Acoust. XCIII: 145-188.

Vardaraj, K. and T.J. Pandian. 1987. Masculinization of *Oreochromis mossambicus* by administration of 17α-methyl-5-androsten-3β-17β-diol through rearing water. Curr.Sci. 56: 412-413.

Wallach, D.E.H., D.M. Surgenor, J. Soderberg and E. Delano. 1959. Preparation and properties of 3,6-dihydroxy-2,4-bis[N-N'-di(carbomethyl)-amino-methyl] fluorescein) utilization of the ultraelectrode termination of calcium. Anal. Chem. 31:456-460.

Williams, A.R. 1983. Ultrasound: Biological effects and potential hazards. Academic Press, New York. 432pp.

Ye, Yimin. 1999. Historical consumption and future demands for fish and fisheries products: exploratory calculations for the years 2015/2030. FAO Fisheries Circular 946,32pp, FAO Rome, Italy.

Zohar, Y., A. D'Emanuele, J. Kost and R.S. Langer. 1991. Ultrasound-mediated administration of compounds into aquatic animals. United State patent No.5,076,208.

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