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(73) Patenthaver:
University of Maryland at Baltimore County, 1000 Hilltop Circle Baltimore MD Maryland 21250, USA

(72) Opfinder:
Yonathan Zohar, 2712 Old Court Road Baltimore 21208 MD Maryland, USA
Ten-tsao Wong, 9 W. Main Blvd. Timonium 21093 MD Maryland, USA

(74) Fuldmægtig:
AWA Denmark A/S, Strandgade 56, 1401 København K, Danmark

(54) Titel: **METHOD AND COMPOSITION HAVING A TRANSPORTER MOLECULE CONJUGATED TO AN ANTI-SENSE MORPHOLINO OLIGOMER FOR SUPPRESSING EXPRESSION OF FISH DEAD END GENE**

(56) Fremdragne publikationer:
XU J. et al.: "Defining Global Gene Expression Changes of the Hypothalamic-Pituitary-Gonadal Axis in Female sGnRH-Antisense Transgenic Common Carp (Cyprinus carpio)" PUBLISHED ONLINE: PLoS ONE | www.plosone.org (2011), Vol. 6(6), pages 1-12.
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(57) Sammendrag:
Methods for the production of reproductively sterile fish and aquatic animals for aquaculture, the aquarium trade, and control of invasive species are described. The methods include disruption of gonadal development through the administration of compounds that lead to the failure of fertile gonadal development. Compounds may be delivered to the eggs prior to fertilization or water activation or post fertilization and water activation by contacting unfertilized or pre-wateractivated fertilized eggs or fertilized eggs in an immersion medium including the compound of interest. Compounds may be conjugated with a molecular transporter compound effective for chorionic transport of the conjugate. The compounds may

be antisense Morpholino oligomers that are capable of effectively suppressing the expression of the dead end gene or other essential genes for germ cell development in fish and other egg-producing aquatic animals.

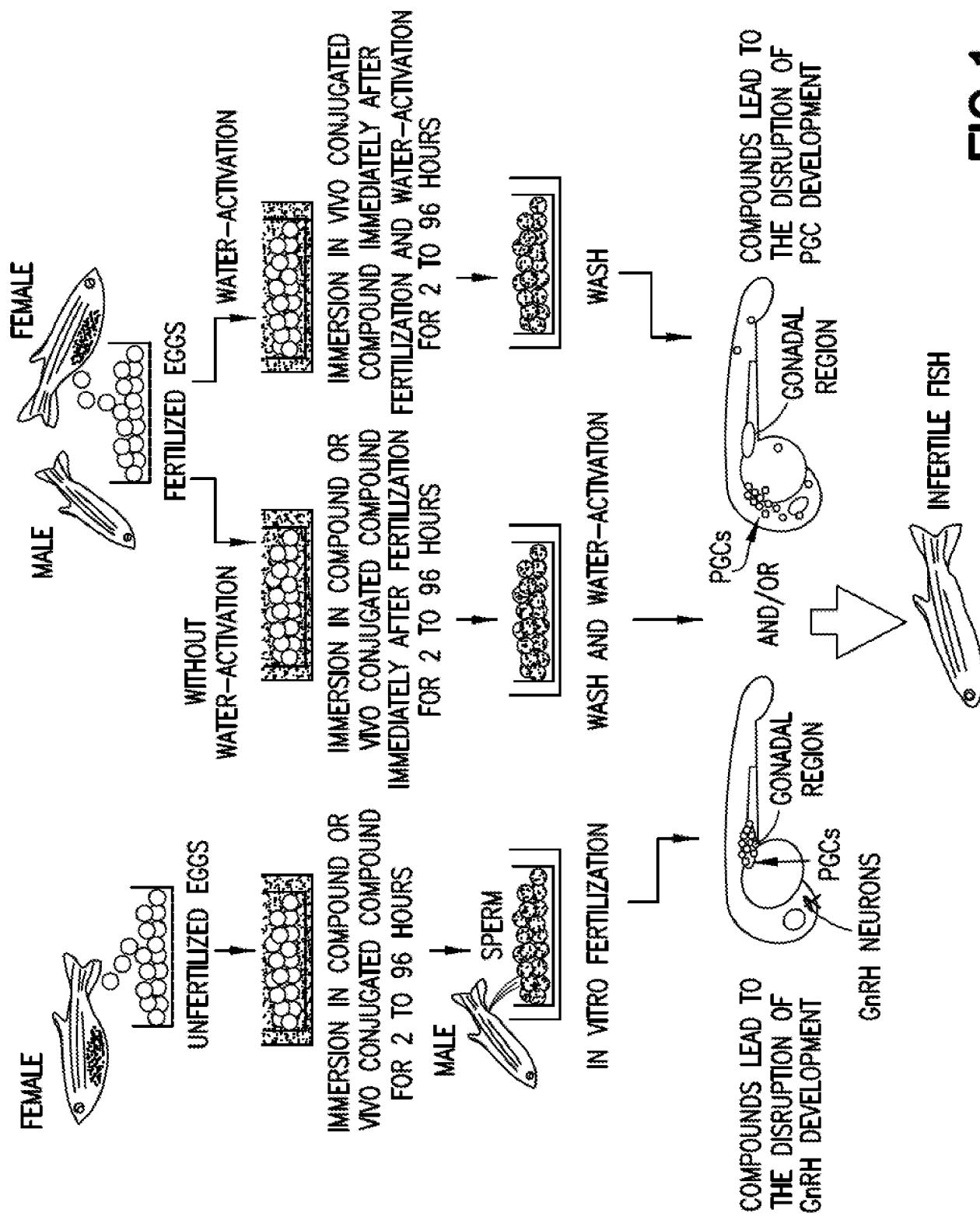


FIG. 1

METHOD AND COMPOSITION HAVING A TRANSPORTER MOLECULE CONJUGATED TO AN ANTI- SENSE MORPHOLINO OLIGOMER FOR SUPPRESSING EXPRESSION OF FISH DEAD END GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to U.S. Provisional Application No. 61/904,652 filed November 15, 2013, U.S. Provisional Application No. 61/968,458 filed March 21, 2014, and U.S. Provisional Application 62/050,815 filed September 16, 2014.

TECHNICAL FIELD

[0002] This disclosure relates to methods for the production of reproductively sterile fish for aquaculture, the aquarium trade, and control of invasive species. The methods include disruption of gonadal development through the administration of compounds that lead to the failure of fertile gonadal development, namely anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene. Such compounds may be delivered to the eggs prior to or after fertilization (or water activation) by contacting by immersion unfertilized or fertilized eggs with an immersion medium comprising the compound of interest.

BACKGROUND ART

[0003] Aquaculture is becoming increasingly important to resolve the current and projected global shortfalls in aquatic foods and seafood availability. As the shift in dependence from fisheries' harvests to artificially propagated aquatic species continues, optimization of aquaculture methods is increasingly necessary to maximize food production and minimize ecological impact, thereby achieving long-term environmental sustainability of our seafood supplies.

[0004] Sterilization (induced infertility) of farmed fish and other egg-producing aquatic animals enhances their growth rate by increasing the conversion of food energy to muscle growth, instead of gonadal development. In addition, if escaped from aquaculture operations to the environment, reproductively sterile farmed fish and other egg-producing aquatic animals, including domesticated, non-native or genetically modified species, will not be able to reproduce or inter-breed with wild population. This will assist biological containment and prevent genetic

contamination of wild populations and/or the establishment in the wild of domestic, non-native or genetically modified farmed fish and other egg-producing aquatic animals.

[0005] Additionally, reproductive sterilization of fish and other egg-producing aquatic animals prevents unauthorized breeding and sale of patented, or otherwise protected, genetically selected or modified fish and other egg-producing aquatic animals.

[0006] XU J. *et al.* (2011), PLoS ONE, Vol. 6(6), discloses a method for inducing infertility in carp based on injection of a composition into fertilized fish eggs. The composition for inducing infertility comprises a DNA plasmid which comprises an antisense DNA fragment targeting sGnRH mRNA. Neither the DNA plasmid nor the antisense DNA fragment contained therein are conjugated to a molecular transporter.

SUMMARY

[0007] The disclosure relates to methods of producing populations of sterile fish, wherein the sterilization methods include disruption of primordial germ cell migration and/or development in each treated individual without detrimentally affecting other characteristics of a normal animal.

[0008] One aspect of the disclosure relates to a method for producing reproductively sterile fish, said method comprising contacting unfertilized egg(s) or fertilized egg(s) prior to water activation in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene that is effective to transfect the egg(s) and render individual(s) produced therefrom reproductively sterile.

[0009] Yet another aspect of the disclosure relates to a method for producing reproductively sterile fish, said method comprising contacting unfertilized egg(s) or fertilized egg(s) prior to water activation in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene that is effective to transfect the egg(s) and render individual(s) produced therefrom reproductively sterile, wherein the antisense Morpholino oligomer is conjugated with a molecular transporter compound effective for chorionic transport of the conjugate. The molecular transporter compound comprises 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidoethyl)aminocarbonyloxyethyl]amino}triazine.

[0010] In another aspect, the disclosure relates to a method for producing reproductively sterile fish, said method comprising contacting fertilized egg(s) in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene conjugated with a

molecular transporter compound that is effective to transfect the fertilized egg(s) and render individual(s) produced therefrom reproductively sterile. The molecular transporter compound comprises 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidoethyl)aminocarbonyloxyethyl]amino}triazine.

[0011] In a further aspect of the disclosure, a method for delivery of compounds into egg(s) from fish is provided comprising contacting by immersion unfertilized or pre-water- activated fertilized egg(s) in an immersion medium with a biologically beneficial compound or a conjugate of a biologically beneficial compound and a molecular transporter compound comprising 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidoethyl)aminocarbonyloxyethyl]amino}triazine. The biologically beneficial compound is an anti-sense Morpholino oligomer which suppresses the expression of the *dead end* gene.

[0012] An additional aspect of the disclosure relates to a composition for treatment of fish egg(s) to render fish produced therefrom reproductively sterile, said composition comprising an anti-sense Morpholino oligomer comprising an oligomer selected from the group consisting of oligomers of SEQ ID NOS: 1-4 and variants thereof that are effective in reproductively sterilizing fish upon contact with fish egg(s).

[0013] In a further aspect, the disclosure relates to a composition wherein the antisense Morpholino oligomer is conjugated with a molecular transporter compound effective for chorionic transport of the conjugate, wherein the molecular transporter compound comprises 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidoethyl)aminocarbonyloxyethyl]amino}triazine.

[0014] Other aspects, features and advantages of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1 is flow chart diagram of an immersion method according to embodiments of the disclosure.

[0016] FIG. 2 is a flow chart diagram of the production of reproductively sterile fish obtained according to other embodiments of the disclosure.

[0017] FIG. 3 is a photomicrograph showing the results of the immersion of zebrafish embryos in an immersion bath of the molecular transporter Vivo conjugated to *dnd*-MO. In FIG. 3A, uncharacterized aggregates were found in fish embryos after 3 hour immersion with 20 μ M *zf**dnd*-MO-Vivo. In FIG. 3B, more aggregates appeared after 5 hour immersion. In FIG. 3C and FIG. 3D, aggregates were not found in the embryos immersed with 20 μ M *zf**dnd*-MO for (3C) 3 hours and (3D) 5 hours (without Vivo).

[0018] FIG. 4 is a fluorescence photomicrograph showing the effects of the administration to fish embryos of a molecular transporter conjugated to an antisense Morpholino oligomer (MO) that effectively blocks the translation of Dead end (Dnd) protein. FIG. 4A shows that in control fish, germ cells migrated to the gonadal region and maintained their morphology. FIG. 4B shows that treatment of *zf**dnd*-MO-Vivo caused germ cell mis-migration and eventually differentiated into other cell types.

[0019] FIG. 5 is a photograph and graphical representation showing zebrafish *dnd*-MO-Vivo treated embryos developed into infertile male-like adults. FIG. 5A shows no difference in appearance or overall size observed between treated adult fish and wild-type males. FIG. 5B shows no significant difference in body-weight of 3-month-old fish ($n = 12$ by random sampling) among *zf**dnd*-MO-Vivo treated fish, and untreated wild-type males.

[0020] FIG. 6 is a photomicrograph showing *zf**dnd*-MO-Vivo induced sterility in zebrafish. Examination of gonadal tissue show (FIG. 6A) well-developed testis of untreated male fish; (FIG. 6B) well-developed ovary of untreated female fish; (FIG. 6C) the gonads of *zf**dnd*-MO-Vivo treated fish developed into a thin filament-like tissue. Photomicrographs (6D-6F) show (FIG. 6D) active spermatogenesis of the testis of untreated male fish; (FIG. 6E) a well-developed ovary of an untreated female fish with oocytes at different developmental stages; (FIG. 6F) the gonad of treated fish appears to be under-developed and surrounded with a large amount of adipocytes without advanced gonadal structure or germ cells.

[0021] FIG. 7 is a graphical representation of zebrafish *dnd*-MO-Vivo treated embryos developed into infertile adults. In both FIG. 7A, 24 hour, and FIG. 7B, 5 to 6 hour immersions, all the embryos that were initially immersed with 60 or 40 μ M of *zf**dnd*-MO-Vivo developed into infertile fish.

[0022] FIG. 8 is a graphical representation of zebrafish *dnd*-MO-Vivo treated embryos developed into infertile adults. In several optimized conditions, when the immersion was initiated immediately after fertilization and lasted to both 24 hour (or prim-5 stage) and 5 to 6

hour (or 50%-epiboly stage), all the embryos that were initially immersed with 60 or 40 μ M of *zfdnd*-MO-Vivo developed into infertile fish.

[0023] FIG. 9 is a photomicrograph showing that eggs are more permeable before water activation. In a 5 hour immersion with 40 μ M of *zfdnd*-MO-Flu, shown in FIG. 9A, unfertilized eggs up-take more *zfdnd*-MO-Flu (stronger green fluorescence) than as shown in FIG. 9B, pre-water-activated eggs.

[0024] FIG. 10 illustrates the portion of the fish used for RNA extraction and the results obtained. Salmonids (trout) eggs were immersed with salmonid's *dnd*-MO at A: Control; B: 10 μ M *Ssdnd*-MO; C: 10 μ M *Ssdnd*-MO +1 μ M *Ssdnd*-MO-Vivo; D: 10 μ M *Ssdnd*-MO +2 μ M *Ssdnd*-MO-Vivo for 48 hours before fertilization. When eggs were treated with 10 μ M *Ssdnd*-MO +1 μ M *Ssdnd*-MO-Vivo or 10 μ M *Ssdnd*-MO +2 μ M *Ssdnd*-MO-Vivo, germ cells were eliminated indicated by the lack of expression of germ cell specific maker gene *vasa*

DETAILED DESCRIPTION OF THE DISCLOSURE

[0025] The present disclosure relates in one aspect to the delivery of beneficial compounds, namely anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene, to eggs from fish by contacting by immersion the eggs in an immersion medium with the compounds prior to fertilization or water activation. Delivery of beneficial compounds into fish has traditionally been achieved via the feed, injection, or immersion of embryos or individuals in a compound of interest. Injection of stock, however, is not practical in large-scale commercial aquaculture operations. In addition, use of immersion treatment of fertilized and water-activated eggs has been limited, due to low permeability of the chorion of the egg, a thick acellular multi-layer coat, also known as the egg envelope, composed mainly of proteins and glycoproteins. Typically, in immersion treatment of fish embryos, large molecular compounds are not able to traverse the chorion and reach the embryo.

[0026] After ovulation/spawning and prior to fertilization and water-activation, eggs have a permeable and perforated chorion (or outermost coat) that allows for entry of water and substances into the unfertilized eggs through the pores or the micropyle, a small canal in the chorion of the egg allowing for the sperm to penetrate the egg for fertilization. Following fertilization and water-activation, the chorion becomes sealed and the egg is rendered impermeable, preventing further uptake of substances or water from the environment.

[0027] As has been discovered, during the window of time between ovulation/spawning and fertilization/water-activation, the eggs are permeable and uptake of compounds from immersion media into the eggs is efficient. Following a short period of immersion, the eggs are fertilized and water-activated at which time they become impermeable and start embryonic development.

[0028] The present disclosure in some aspects describes methods for efficiently delivering anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene into eggs of fish by contacting the eggs with the anti-sense Morpholino oligomer during a window of time when the eggs are permeable to environmental substances, i.e., after ovulation/spawning and before fertilization or after fertilization and before water activation. The contacting comprises immersion of the eggs in an immersion medium containing one or more such anti-sense Morpholino oligomers.

[0029] Accordingly, in various aspects, the present disclosure relates to contacting unfertilized eggs or fertilized eggs prior water-activation with a compound, namely an anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene, in immersion media. The immersion medium in specific embodiments may include additional compounds or other materials that are beneficial to the fish hatched from eggs contacted with the immersion medium, e.g., materials such as DNA/RNA, hormones, growth promoters, protective antigens, nutrients, etc.

[0030] The disclosure thus contemplates methods of producing reproductively sterile fish involving contacting eggs in immersion medium with said selected compounds resulting in reproductively sterile individuals. The sterilization methods comprise the disruption of gonadal development in the embryo. The present disclosure also relates to methods of preventing interbreeding between domesticated, non-native and genetically modified farmed fish/other fish and their wild stocks, as well as to the establishment of such aquacultured fish and other fish in the wild. In addition, the disclosed methods may be employed to enable prevention of genetic contamination of a wild population by farmed fish and other fish.

[0031] The methods of the disclosure are applicable to fish.

[0032] Accordingly, fish includes all fish species, including salmon, Atlantic salmon, coho salmon, chinook salmon, chum salmon, sockeye salmon, pink salmon, masu salmon, trout, rainbow trout, brook trout, brown trout, common grayling, Arctic grayling, Arctic char, bass, hybrid bass, striped bass, white bass, striped-white bass hybrids, yellow bass, perch, white perch, yellow perch, European perch, bass-perch hybrids, Nile tilapia, blue tilapia, blue-Nile

tilapia hybrids, Mozambique tilapia, zebrafish, carp species, breams, seabreams, porgies, catfish species, and cod.

[0033]

[0034] As defined herein, "sterilizing" fish is understood to mean rendering an individual unable to sexually reproduce. Reproductively sterile fish are defined as individuals that are unable to reach sexual maturity or to reproduce when reaching the age of sexual maturity.

[0035] The methods of producing a reproductively sterile fish include administration of compounds, i.e. anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene to their eggs prior to fertilization or water-activation to disrupt gonadotropin releasing hormone (GnRH) cell development and/or primordial germ cell (PGC) development, migration and colonization in the gonad of the embryo, which results in failure of gonad development and/or failure of full and proper gonadal functioning at the cellular or tissular level, and ultimately the generation of sterile fish.

[0036] GnRH is required for gonadal development and the maintenance of reproductive cycles in vertebrates. Specifically, GnRH, also known as luteinizing hormone releasing hormone (LHRH), stimulates the synthesis and secretion of the gonadotropins, in particular follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that are essential to gonadal development and, therefore, disruption of the synthesis and secretion of GnRH is a potent method to induce sterility.

[0037] PGCs are a population of cells in the fish embryo that are precursors of the gametes of the adult fish. The PGCs are produced during the very early stages of embryonic development. At later stages of embryonic development, the PGCs migrate through the embryo from their original location to the area of the gonadal precursors. At the end of their migration, the PGCs enter the developing gonads, colonize the tissue and start the process of gametogenesis, leading to mature gonads in the adult fish.

[0038] The methods of the disclosure allow generation of reproductively sterile (infertile) fish. The sterilization strategy will specifically disrupt gonad development in the individuals without detrimentally affecting any other characteristics resulting in the production of completely normal but reproductively sterile fish.

[0039] FIG. 1 is a diagram of the immersion method of embodiments of the disclosure. As shown, compounds are delivered to the eggs by immersing unfertilized eggs (or pre-water-activated eggs) or fertilized eggs in the compounds of interest, i.e. in the context of the

invention, an anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene, in an immersion media. Typically, the eggs are immersed in the compound for 2 to 96 hours or more depending on the species and immersion temperature. The administration of the compound will preferably take place while the chorion of the eggs is permeable. The treatment with the compounds of interest will result in early stage embryos having disruption of GnRH development or PGC development. When eggs are treated by the immersion process, the resulting adult fish will be reproductively infertile or sterile, typically having no fertile gonadal development.

[0040] Thus, in various embodiments, the disclosure provides a method to efficiently administer compounds, i.e. the aforementioned anti-sense Morpholino oligomers, into embryos by immersing eggs in an immersion medium with compounds prior to fertilization or water-activation. The selected compounds disrupt GnRH cell and/or PGC development, migration and/or survival in large numbers of embryos, resulting in large-scale production of reproductively sterile adult fish. The methods of the disclosure are also applicable to single embryos in smaller scale production of reproductively sterile adult fish.

[0041] The anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene for use in the methods of the disclosure are compounds known to disrupt GnRH cell and/or PGC development, migration and/or survival which are capable of entering the chorion of a pre-fertilized or a pre-water-activated egg. Thus, antisense Morpholino oligomer useful in the methods of the disclosure is anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene that is effective to transfect the egg(s) and render individual(s) produced therefrom reproductively sterile.

[0042] Antisense Morpholino oligomer is used to transiently silence gene expression by either blocking translation or RNA splicing that is an essential step to generate mRNA. Specific antisense Morpholino oligomers can be identified to transiently block or suppress the expression of *deadend* which results in the failure of gonadal development and ultimately generates sterile fish.

[0043] Thus, in one aspect of the disclosure, a method for producing reproductively sterile fish is provided comprising contacting, i.e. immersing, unfertilized egg(s) or pre-water-activated fertilized eggs in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene that is effective to transfect the egg(s) and render individual(s)

produced therefrom reproductively sterile. The contacting comprises chorionic transfection of the egg(s).

[0044] In such aspect, the disclosure relates to methods of producing reproductively sterile fish by administration of effective Morpholino oligomers suppressing the expression of the *dead end* gene to eggs in order to disrupt primordial germ cell (PGC) development, and migration to, and colonization in, the gonad of the embryo, which results in the failure of gonad development and/or full and proper gonadal functioning at the cellular or tissular level, and ultimately the generation of sterile fish.

[0045] *Dead end (dnd)* is a vertebrate-specific component of the germ plasm and germ-cell granules that is essential for germ cell development in certain fish. The *dnd* gene is specifically expressed in germ plasm and primordial germ cells. Since *dnd* is considered essential for normal migration and survival of PGCs, embryos devoid of this protein develop to become sterile adults.

[0046] The disclosed methods are useful for the production of reproductively sterile fish for aquaculture, the aquarium trade, and control of invasive species. In one aspect, the methods include disruption of gonadal development through the administration of antisense Morpholino oligomer against *dead end* mRNA (*dnd*-MO) to pre-fertilized or pre-water-activated egg(s). The action of *dnd*-MO leads to the failure of fertile gonad development and to sterile adult fish.

[0047] In embodiments, the *dnd*-MO is able to transiently suppress expression of *dead end* protein that is essential for embryonic germ cell development.

[0048] The present disclosure also relates to specific Morpholino oligomer sequences for use in methods for suppression of expression a *dead end* gene in a fish.

[0049] FIG. 2 is a flow chart for production of reproductively sterile fish by the administration of specific Morpholino oligomers against the *dead end* mRNAs of salmonids, moronids or cichlids to disrupt primordial germ cell (PGC) development, which results in the failure of gonad development, and ultimately the generation of sterile fish. When eggs are not treated with Morpholino oligomers, they may become fertile broodstock.

[0050] As shown in FIG. 2, eggs from salmonids, moronids or cichlids, or other fish species, may be contacted with Morpholino oligomers against the *dead end* genes of the relevant fish species. FIG. 2 illustrates the administration of specific Morpholino oligomers against the *dead end* genes of salmonids, moronids or chichlids. Alternatively, the eggs may be given no treatment. As shown, where the unfertilized eggs are contacted with Morpholino oligomers,

oligomers effect the suppression of or blocking of Dead end protein translation, resulting in the disruption of PCG development. The adult salmonids, monoids or cichlids are consequently infertile, since there is no fertile gonadal development. When normal PGC development is permitted, the fish will have normal fertile gonadal development and the fish may be used as broodstock.

[0051] Thus, in embodiments, the antisense Morpholino oligomers are capable of effectively suppressing expression of at least one of Salmonidae *dead end* gene, Moronidae *dead end* gene or cichlidae *dead end* gene.

[0052] In another aspect, the disclosure relates to specific antisense Morpholino oligomers that are able to transiently and effectively suppress the translation of Dead end, an essential protein for germ cell survival, and specifically disrupt gonadal development resulting in the production of infertile fish, e.g., salmonids (salmons and trouts), moronids (basses) and cichlids (tilapias and ornamental cichlids).

[0053] In another aspect, the disclosure relates to a specific Morpholino oligomer, 5'-CTGACTTGAACGCTCCTCCATTATC-3' (SEQ ID NO: 1) and its variants, e.g., 5'-ACTTGAACGCTCCTCCAT-3' (SEQ ID NO: 2), that are able to transiently and effectively suppress the expression of Salmonidae *dead end* gene, which results in the failure of gonad development and/or the failure of full and proper gonadal functioning, and ultimately the generation of sterile salmonids. Accordingly, the methods of the disclosure are applicable to all salmonids, including Atlantic, coho, chinook, chum, sockeye, pink and masu salmons, rainbow, brook and brown trouts, common and Arctic grayling, and Arctic charr, among others.

[0054] In another aspect, the disclosure relates to a specific Morpholino oligomer, 5'-GGCTCTGCTTGCTCTCCATCATCTC-3' (SEQ ID NO: 3) and its variants that are able to transiently and effectively suppress the expression of Moronidae *dead end* gene, which results in the failure of gonad development and/or full and proper gonadal functioning, and ultimately the generation of sterile moronids. Accordingly, the methods of the disclosure are applicable to all moronids including striped bass, white bass, striped-white bass hybrids, yellow bass, white perch, yellow perch, European perch, and bass-perch hybrids, among others.

[0055] In another aspect, the disclosure relates to a specific Morpholino oligomer, 5'-CTGGCTTGCGTGTTCATCGTC-3' (SEQ ID NO: 4) and its variants that are able to transiently and effectively suppress the expression of cichlidae *dead end* gene, which results in the failure of gonad development and/or the failure of full and proper gonadal functioning, and

ultimately the generation of sterile cichlids. Accordingly, the methods of the disclosure are applicable to all cichlid and tilapia species, including Nile tilapia, blue tilapia, blue-Nile tilapia hybrids, Mozambique tilapia, and other edible and ornamental cichlid species and their hybrids.

[0056] The Morpholino oligomers may be variants of the listed sequences. These variations may include other modified nucleic acids and other Morpholino oligomers that cover the whole or partial sequences listed above. Particularly included are antisense oligomers that comprise, consist essentially of, or consist of, one or more of SEQ ID NOS:1, 3 and 4. Also included are variants of these antisense oligomers, including variant oligomers having 80%, 85%, 90%, 95%, 97%, 98%, or 99% (including all integers in between) sequence identity or sequence homology to any one of SEQ ID NOS: 1, 3 and 4, and/or variants that differ from these sequences by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides, preferably variants that are effective in reproductively sterilizing fish upon contact with fish egg(s). SEQ ID NO:2 is a preferred variant of SEQ ID NO: 1.

[0057] Variants that are effective in reproductively sterilizing fish upon contact with fish egg(s) as used in such context means use of the variants in the methods disclosed herein will result in reproductively sterile fish. In embodiments, the variants will effectively suppress the expression of a *dead end* gene of interest.

[0058] According to embodiments of the methods of the disclosure, unfertilized fish egg(s) (one or more eggs) are immersed in an immersion medium comprising an antisense Morpholino oligomer capable of effectively suppressing the expression of a *dead end* gene in the fish of interest. The concentration of the antisense Morpholino oligomer in the bath should be sufficient to allow the antisense Morpholino oligomer to traverse the chorion of the fish egg(s), effectively transfecting the egg(s). In embodiments, such concentration will typically be 1 to 20, more preferably, 3 to 15 μ M, and still more preferably, 5 to 10 μ M.

[0059] The immersion media is an aqueous medium which may further comprise fish ovarian fluid or fertilization diluent that contains salt, Tris (pH 7-9), Glycine, and/or 0 to 30% of serum and protease inhibitors such as aprotinin or leupeptin.

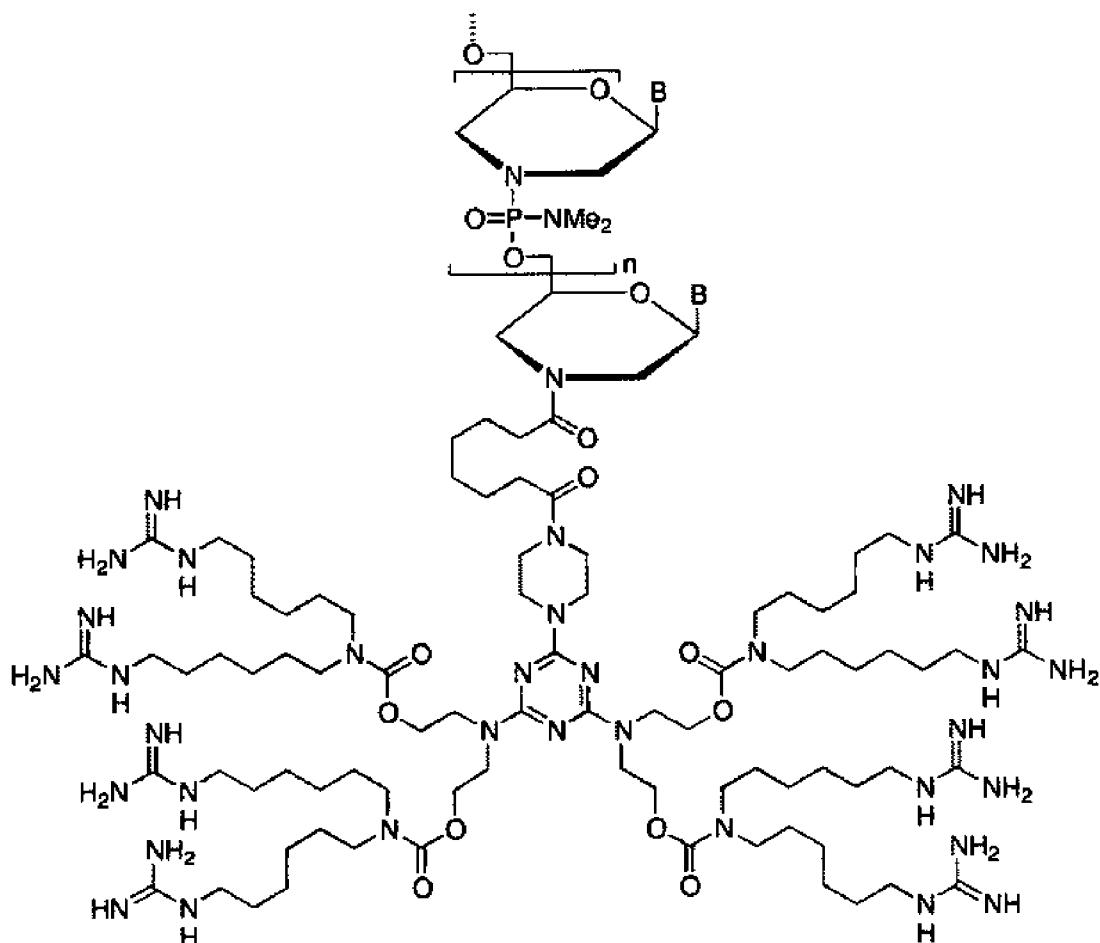
[0060] Although the time required for the immersion of the unfertilized eggs or pre-water-activated fertilized eggs to result in satisfactory sterilization of the fish will depend on the species of fish, typically the fish eggs will be immersed in the immersion media containing a Morpholino oligomer for 2 to 96 hours, more preferably for 4 to 72 hours, and still more preferably from 5 to 48 hours.

[0061] The present disclosure further relates to methods for the production of reproductively sterile fish for aquaculture, the aquarium trade, and control of invasive species wherein the methods include disruption of gonadal development through the administration of molecular transporter-conjugated compounds, i.e. anti-sense Morpholino oligomers suppressing the expression of the *dead end* gene to the egg or embryos, by which the compounds are able to transfect the egg(s), i.e., reach and enter either unfertilized eggs or embryos. The action of the compounds inside of the egg or embryos leads to the failure of fertile gonad development and ultimately to the production of sterile adult fish. The molecular transporter, i.e. 2-[(4-nitrophenyl)oxy carbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido hexyl)aminocarbonyloxyethyl]amino}triazine, is able to effectively transport the compounds conjugated to it through minuscule channels and pores, thereby allowing chemicals, drugs, and peptides to pass through the chorion and enter the egg or embryo and reach the target tissue. Thus, via these methods, eggs, either unfertilized or fertilized, may be effectively contacted by immersion in an immersion medium with said Morpholino oligomers to produce reproductively sterile fish.

[0062] In one aspect, the disclosure relates to methods of producing reproductively sterile fish by administration of the aforementioned molecular transporter-conjugated compounds to either unfertilized fish eggs or embryos in order to disrupt gonadotropin releasing hormone (GnRH) cell development and/or primordial germ cell (PGC) development and migration to, and colonization in, the gonad of the embryo, which results in the failure of gonad development and/or the failure of full and proper gonadal functioning at the cellular or tissular level, and ultimately the generation of sterile fish

[0063] In one aspect, the disclosure relates to a molecular transporter comprising 2-[(4-nitrophenyl)oxy carbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido hexyl)aminocarbonyloxyethyl]amino}triazine as described in U.S. Patent No. 7,935,816. Such molecular transporter is also known in the art as "Vivo".

[0064] 2-[(4-nitrophenyl)oxy carbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido hexyl)aminocarbonyloxyethyl]amino}triazine with morpholino as a representative bioactive substance is shown in the following conjugate:



[0065]

The disclosure further relates to the use of 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidohexyl)aminocarbonyloxyethyl]amino}triazine as a molecular transporter to enable chorionic transport of a conjugate of the molecular transporter compound and Morpholino oligomer suppressing the expression of the *dead end* gene in fish.

[0066] In another aspect of the disclosure, methods are provided for delivery of compounds into egg(s) from fish comprising contacting by immersion of unfertilized or pre-water-activated egg(s) in an immersion medium with at least one biologically beneficial compound. According to these methods, the unfertilized or pre-water-activated egg(s) are sufficiently permeable to effectively uptake the at least one biologically beneficial compound. "Effectively uptake" as used herein means that the biologically beneficial compound will be effective for the beneficial effect intended by use thereof. The biologically beneficial compound is an anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene.

[0067] In embodiments, the biologically beneficial compound will be provided as a conjugate of a biologically beneficial compound and a molecular transporter compound comprising 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidohexyl)aminocarbonyloxyethyl]amino}triazine. In these embodiments the molecular transporter compound is effective for chorionic transport of the biologically beneficial compound such that the contacting, i.e. immersion in an immersion medium, comprises chorionic transfection of the egg(s). The biologically beneficial compound, in embodiments providing the compound in a conjugate form, is a compound which can form a conjugate with the molecular transporter compound. The biologically beneficial is an anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene.

[0068] In another aspect, the disclosure provides a method for producing reproductively sterile fish, said method comprising contacting by immersion unfertilized egg(s) or pre-water-activated fertilized eggs in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene that is effective to transfect the egg(s) and render individual(s) produced therefrom reproductively sterile, wherein the anti-sense Morpholino oligomer is conjugated with a molecular transporter compound comprising 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidohexyl)aminocarbonyloxyethyl]amino}triazine effective for chorionic transport of the conjugate.

[0069] In another aspect, the disclosure contemplates a method for producing reproductively sterile fish, said method comprising contacting by immersion fertilized egg(s) (or embryos) in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene conjugated with a molecular transporter compound comprising 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidohexyl)aminocarbonyloxyethyl]amino}triazine that is effective to transfect the egg(s) and render individual(s) produced therefrom reproductively sterile.

[0070] The selected oligomers may disrupt GnRH cell and/or PGC development, migration and/or survival in large numbers of embryos, resulting in large-scale production of reproductively sterile adult fish. The methods of the disclosure are also applicable to single embryos in smaller scale production of reproductively sterile adult fish.

[0071] In embodiments, the methods include preparation and use of the aforementioned molecular transporter conjugated with an antisense Morpholino oligomer capable of effectively

suppressing the expression of *dnd* in fish species. In other embodiments, the antisense Morpholino oligomer is capable of effectively suppressing the expression of at least one of Salmonidae *dead end* gene, Moronidae *dead end* gene or cichlid *dead end* gene. In further embodiments, the antisense Morpholino oligomer is SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 or variants thereof which are capable of effectively suppressing the expression of *dead end* in fish species.

[0072] In one embodiment, a method for producing reproductively sterile fish is provided, comprising immersing unfertilized or fertilized fish egg(s) in an immersion media comprising a conjugate of a molecular transporter compound and an antisense Morpholino oligomer capable of effectively suppressing expression of the *dead end* gene that is essential for gonadal development in the fish. The molecular transporter is 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido-hexyl)aminocarbonyloxyethyl]amino}triazine.

[0073] According to particular aspects of the methods of the disclosure, unfertilized egg(s) or pre-water-activated egg(s) (one or more) are immersed in an aqueous medium comprising an antisense Morpholino oligomer or an antisense Morpholino oligomer-transporter conjugate capable of effectively suppressing the expression of a *dead end* gene. The molecular transporter is 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di[di(trifluoroacetamidohexyl)aminocarbonyloxyethyl]amino}triazine. The concentration of the antisense Morpholino oligomer in the bath should be sufficient to allow the antisense Morpholino oligomer-transporter conjugate to traverse the chorion of the egg(s). In embodiments, such concentration will typically be 1 to 20 μ M, more preferably 3 to 15 μ M, even more preferably from 5 to 10 μ M.

[0074] Alternatively, the disclosure contemplates a method for producing reproductively sterile fish, said method comprising contacting by immersion fertilized egg(s) in an immersion medium with anti-sense Morpholino oligomer suppressing the expression of the *dead end* gene conjugated with a molecular transporter compound that is effective to transfect the fertilized egg(s) and render individual(s) produced therefrom reproductively sterile. The contacting comprises chorionic transfection of the egg(s). The molecular transporter is 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido-hexyl)aminocarbonyloxyethyl]amino}triazine.

[0075] In embodiments of this method, fertilized eggs (embryos) are treated by immersion in an immersion medium comprising an antisense Morpholino oligomer-transporter conjugate capable of effectively suppressing the expression of a *dead end* gene. The concentration of the antisense Morpholino oligomer in the bath should be sufficient to allow the antisense Morpholino oligomer-transporter conjugate to traverse the chorion of the fertilized egg(s). Such concentration will be higher than the amount needed for immersion of unfertilized fish egg(s) since, after fertilization and water-activation, the egg chorion becomes impermeable, hindering traversal into the egg. Typically, the concentration of the bath for immersion of embryos is 20 to 80 μ M, preferably 40 to 60 μ M.

[0076] In embodiments of the treatment of fertilized eggs, the antisense Morpholino oligomer is capable of effectively suppressing the expression of at least one of Salmonidae *dead end* gene, Moronidae *dead end* gene or cichlid *dead end* gene. In further embodiments, the antisense Morpholino oligomer is SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 or a variant thereof which is capable of effectively suppressing the expression of *dead end* in the fish species.

[0077] In another aspect of the disclosure, a composition effective for disruption of primordial germ cell migration in fish is provided, comprising an antisense Morpholino oligomer selected from SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 4 or variants such as SEQ ID NO: 2 thereof which are capable of effectively suppressing expression of the *dead end* gene in fish. In such aspect, a composition for treatment of fish egg(s) to render fish produced therefrom reproductively sterile is provided, comprising an anti-sense Morpholino oligomer comprising an oligomer selected from the group consisting of oligomers of SEQ ID NOS: 1-4 and variants thereof that are effective in reproductively sterilizing fish upon contact with fish egg(s). This Morpholino oligomer composition preferably is used in treatment of unfertilized fish egg(s).

[0078] The disclosure further relates to a novel compound entity that is able to specifically disrupt gonadal development resulting in the production of infertile fish. The new compound entity comprises a molecular transporter which is 2-[(4-nitrophenyl)oxycarbonylhexamethylenecarbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamido-hexyl)aminocarbonyloxyethyl]amino}triazine conjugated to a Morpholino antisense oligomer that effectively suppresses the translation of *dead end*, an essential protein for germ cell survival, mRNA. Such conjugate may be used for treating unfertilized or pre-water-activated fertilized eggs or for treating fertilized eggs.

[0079] Such conjugated compounds may be used for the production of sterile fish. The morpholino oligomer compounds can be conjugated to the molecular transporter to effectively traverse the chorion and enter the embryos. Hence, the compounds are able to specifically disrupt gonadal development resulting in the generation of infertile fish. Preferably, the molecular transporter comprises 2-[(4-nitrophenyl)oxygenylhexamethylene-carbonylpiperazinyl]-4,6-bis{di-[di(trifluoroacetamidohexy)aminocarbonyloxyethyl]-amino}triazine and the oligomer comprises an antisense Morpholino oligomer selected from SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 4 or any of various variants that are effective in reproductively sterilizing fish upon contact with fish egg(s), such as SEQ ID NO: 2.

[0080] It will be recognized that the contacting of eggs and/or embryos with the compounds, conjugates, and compositions of the present disclosure may be carried out in any suitable manner, i.e., involving immersion contacting. It is to be recognized that immersion contacting provides an efficient and effective contacting technique that is amenable to large-scale operations for the production of reproductively sterile fish.

[0081] The advantages and features of the disclosure are further illustrated with reference to the following examples, which are not to be construed as in any way limiting the scope of the disclosure but rather as illustrative of particular embodiments of the disclosure in specific applications thereof.

[0082] Example 1:

[0083] Zebrafish were selected for initial exemplification of the methods of the disclosure, due to their short generation time and large numbers of embryos produced per mating, which are easily obtained on a daily or weekly basis. Additionally, the embryos of zebrafish are transparent, providing ease of visual observations, and are hardy. The normal development of GnRH cells, PGCs and gonads within the embryo is an evolutionarily conserved mechanism that is found in all fish. Accordingly, the methods of the disclosure are applicable to all fish species, including zebrafish, carp species, trout species, salmon species, breams (including seabreams and porgies), basses (including marine and freshwater seabass and hybrid basses), perches (yellow perch, white perch), catfish species, cod and other major classes that are candidates for captive culture.

[0084] Vivo and *dnd*-MO

[0085] A molecular transporter, a dendrimeric octaguanidine with a triazine core also known as Vivo, was conjugated to zebrafish *dnd*-MO, 5'-GCTGGGCATCCATGTCTCCGACCAT-3'

(SEQ ID NO 5) (zfdnd-MO-Vivo) and used in an immersion containing zebrafish embryos in water. After a 3-hour immersion with 20 μ M of zfdnd-MO-Vivo, uncharacterized aggregates were found around the inner part of chorion or the surface of the blastodisc of the embryos (FIG. 3A). More aggregates appeared after a 5-hour immersion (FIG. 3B). These aggregates were not seen in the non-conjugated control groups that immersed with 20 μ M of zfdnd-MO for 3 (FIG. 3C) or 5 (FIG. 3D) hours. The results indicated that conjugation of the antisense Morpholino oligomers with Vivo provided the ability of the conjugated compound to act on chorion that caused aggregates.

[0086] At 2 days post-fertilization, fluorescence microscopy examination revealed that germ cells in the embryos immersed with 40 or 60 μ M zfdnd-MO-Vivo had mis-migrated and differentiated to other cell types (FIG. 4A, FIG. 4B). As shown, zebrafish dnd-MO-Vivo disrupted germ cell development in zebrafish. FIG. 4A shows that in control fish, germ cells migrated to the gonadal region and maintained their morphology (round-shape cells). FIG. 4B shows that that treatment of zfdnd-MO-Vivo caused germ cell mis-migration and eventual differentiated into other cell types.

[0087] Example 2:

[0088] The zebrafish treated in Example 1 were examined after development into adult fish. The bath immersion of zebrafish embryos with 40 or 60 μ M zfdnd-MO-vivo allowed for efficiently inducing 100% sterility in the individuals that were treated under optimal conditions as shown in FIG. 8, without affecting any other physiological characteristics of the fish (FIG. 5). As shown in FIG. 5, zebrafish *dnd*-MO-Vivo treated embryos developed into infertile male-like adults. FIG. 5A shows no difference in appearance or overall size observed between treated adult fish and wild-type males. FIG. 5B shows no significant difference in body-weight of 3-month-old fish ($n = 12$ by random sampling) among zfdnd-MO-Vivo treated fish, and untreated wild-type males.

[0089] In additional experiments, the methods of the disclosure were successfully applied to zebrafish and the duration of immersion was decreased to 5 to 6 hours without affecting the efficiency of sterility (FIG. 6 FIG. 7, and FIG. 8). In FIG. 6, zfdnd-MO-Vivo induced sterility in zebrafish is shown. Examination of gonadal tissue showing (A) A well-developed testis of untreated male fish. (B) A well-developed ovary of untreated female fish. (C) The gonads of zfdnd-MO-Vivo treated fish developed into a thin filament-like tissue. Photomicrographs (D-F) show (D) active spermatogenesis of the testis of untreated male fish. (E) A well-developed ovary

of an untreated female fish with oocytes at different developmental stages. (F) The gonad of treated fish appears to be under-developed and surrounded with a large amount of adipocytes without advanced gonadal structure or germ cells.

[0090] As shown in FIG. 7 and FIG. 8, zebrafish *dnd*-MO-Vivo treated embryos developed into infertile adults. In both A) 24 hour and B) 5 to 6 hour immersions, all the embryos that were initially immersed with 60 or 40 μ M of *zf**dnd*-MO-Vivo immediately after fertilization developed into infertile fish.

[0091] Example 3:

[0092] To continuously optimize the technology, the bath immersion was conducted using unfertilized eggs since the permeability of the chorion gradually decreases after fertilization and water activation. Our results show that when eggs were immersed with 40 μ M fluorescein labelled *zf**dnd*-MO (*dnd*-MO-Flu), the unfertilized eggs up-took more *dnd*-MO-Flu than the eggs were water-activated 1 hour before immersion (FIG. 9). As shown in FIG. 9, eggs are more permeable before water activation. In a 5 hour immersion with 40 μ M of *zf**dnd*-MO-Flu, A) unfertilized eggs up-take more *zf**dnd*-MO-Flu (stronger green fluorescence) than B) pre-water-activated eggs.

[0093] Example 4:

[0094] When pre-fertilized salmonids (trout) eggs were immersed with salmonid's *dnd*-MO +*dnd*-MO-Vivo (*Ssdnd*-MO +*Ssdnd*-MO-Vivo), germ cells were eliminated (FIG. 10) indicated by the lack of expression of germ cell specific maker gene *vasa*. As shown in FIG. 10, Salmonids (trout) eggs were immersed with salmonid's *dnd*-MO at A: Control; B: 10 μ M *Ssdnd*-MO; C: 10 μ M *Ssdnd*-MO +1 μ M *Ssdnd*-MO-Vivo; D: 10 μ M *Ssdnd*-MO +2 μ M *Ssdnd*-MO-Vivo for 48 hours before fertilization. Soon after hatch, the middle part of body was cut and used for RNA extraction and RT-PCR for detecting *vasa* expression. One fish from group C and one from group D appeared to have very low *vasa* expression, indicating possible effect on reducing germ cell abundance. RT: reverse transcription.

[0095] As described herein, the methods are generally applicable to farmed fish, as production of sterile farmed species is desirable. Accordingly, the methods of the invention are applicable to any farmed species of fish, particularly to commercially important farmed species.

[0096] While the disclosure has been set out herein in reference to specific aspects, features and illustrative embodiments, it will be appreciated that the utility of the disclosure is

not thus limited, but rather extends to and encompasses numerous other variations, modifications and alternative embodiments, as will suggest themselves to those of ordinary skill in the field of the present disclosure, based on the description herein. Correspondingly, the invention as hereinafter claimed is intended to be broadly construed and interpreted, as including all such variations, modifications and alternative embodiments, within its spirit and scope.

INDUSTRIAL APPLICABILITY

[0097] The methods and compounds of the disclosure produce reproductively sterile fish. Sterilization (induced infertility) of farmed fish enhances their growth rate by increasing the conversion of food energy to muscle growth, instead of gonadal development. In addition, if escaped from aquaculture operations to the environment, reproductively sterile farmed fish, including domesticated, non-native or genetically modified species, will not be able to reproduce or inter-breed with wild stock. This will assist biological containment and prevent genetic contamination of wild populations and/or the establishment in the wild of domestic, non-native or genetically modified farmed fish.

P A T E N T K R A V

1. Fremgangsmåde til levering en antisense-morpholino-oligomer som undertrykker ekspression af *dead end*-genet, ind i ubefrugtet/ubefrugtede fiskeæg eller præ-vandaktiveret/præ-vandaktiverede befrugtet/befrugtede fiskeæg eller befrugtet/befrugtede fiskeæg for at gøre fisk produceret derfra reproduktionsmæssigt sterile ved choriontransfektion af ægget/æggene, hvilken fremgangsmåde omfatter at nedsænke ægget/æggene i et nedsænkningsmedium omfattende antisense-morpholino-oligomeren og hvor antisense-morpholino-oligomeren er konjugeret med en molekylær transportør som omfatter 2-[(4-nitrophenyl)oxygenylhexamethylencarbonyl-piperazinyl]-4,6-bis{di[di-(trifluor-acetamidoethyl)-aminocarbonyloxyethyl]amino}triazin, hvis ægget/æggene er befrugtet/befrugtede æg.
2. Fremgangsmåde ifølge krav 1, hvor ægget/æggene er ubefrugtet/ubefrugtede æg eller præ-vandaktiveret/præ-vandaktiverede æg.
3. Fremgangsmåde ifølge krav 2, hvor antisense-morpholino-oligomeren er konjugeret med den molekylære transportør.
4. Fremgangsmåde ifølge krav 1, hvor ægget/æggene er befrugtet/befrugtede æg.
5. Fremgangsmåde ifølge krav 1 til 4, hvor *dead end*-genet omfatter mindst én af Salmonidae, Moronidae og cichlidae *dead end*-gener.
6. Fremgangsmåde ifølge krav 1 til 5, hvor antisense-morpholino-oligomeren omfatter en oligomer valgt fra gruppen bestående af oligomerer ifølge SEQ ID NOS: 1-4 og varianter deraf som er effektive til reproduktionsmæssigt at sterilisere fisk ved kontakt med fiskeægget/fiskeæggene.
7. Fremgangsmåde ifølge krav 6, hvor antisense-morpholino-oligomeren omfatter en oligomer valgt fra gruppen bestående af oligomerer ifølge SEQ ID NOS: 1-4.
8. Sammensætning til behandling af fiskeæg til at gøre fisk produceret

derfra reproduktionsmæssigt sterile, hvilken sammensætning omfatter en molekylær transportørforbindelse, 2-[(4-nitrophenyl)oxycarbonylhexamethylencarbonylpiperazinyl]-4,6-bis{di-[di(trifluoracetamidohexyl)-aminocarbonyloxyethyl]amino}triazin, til hvilken 5 en morpholino-oligomer som har en antisense-sekvens for mRNA for fiske *dead end*-gen, er konjugeret.

9. Sammensætning ifølge krav 8, hvor antisense-sekvensen er valgt fra gruppen bestående af SEQ ID NOS: 1-4 og steriliserende varianter deraf.

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10. Sammensætning ifølge krav 8, hvor antisense-sekvensen er valgt fra gruppen bestående af SEQ ID NOS: 1-4.

1/10

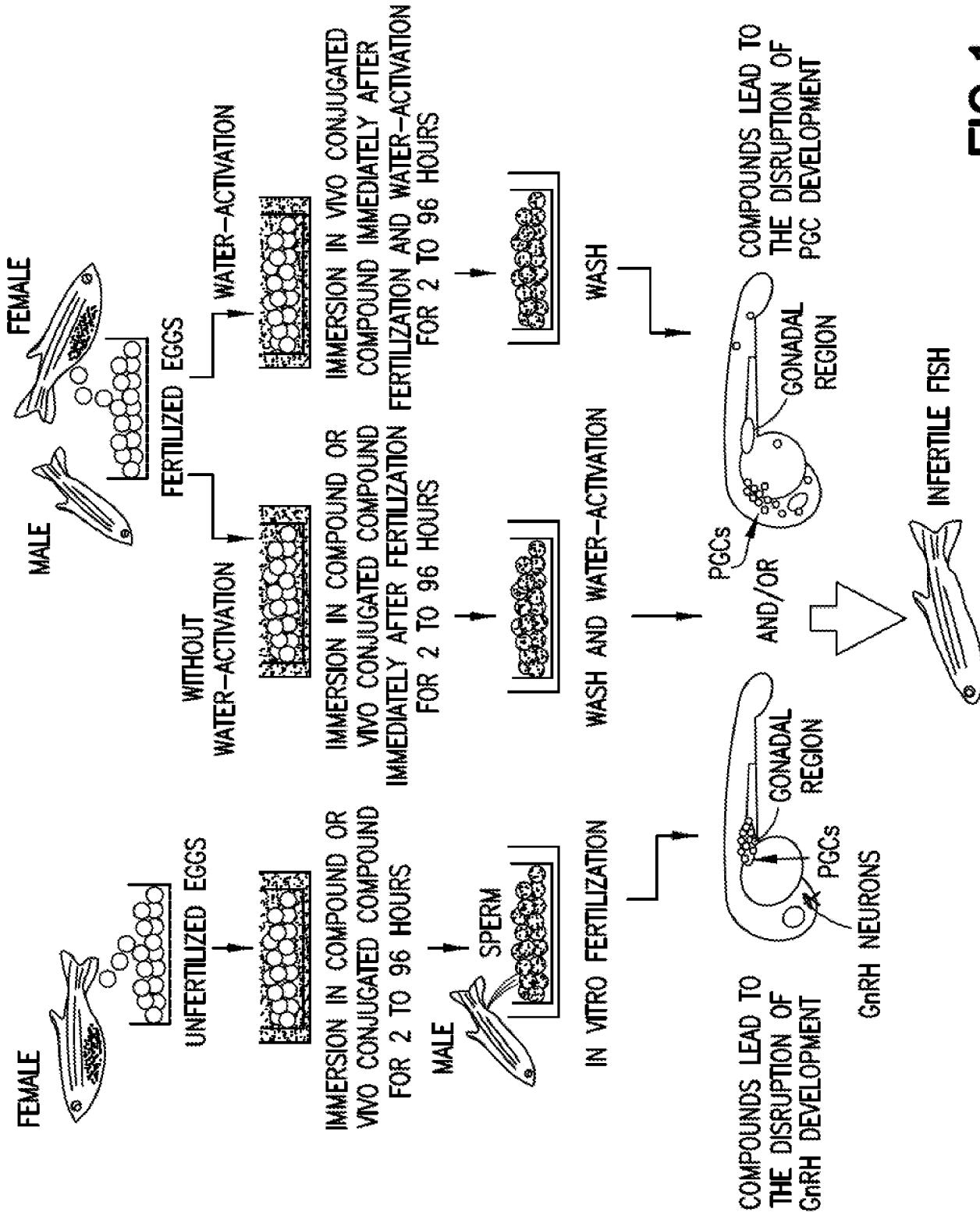


FIG. 1

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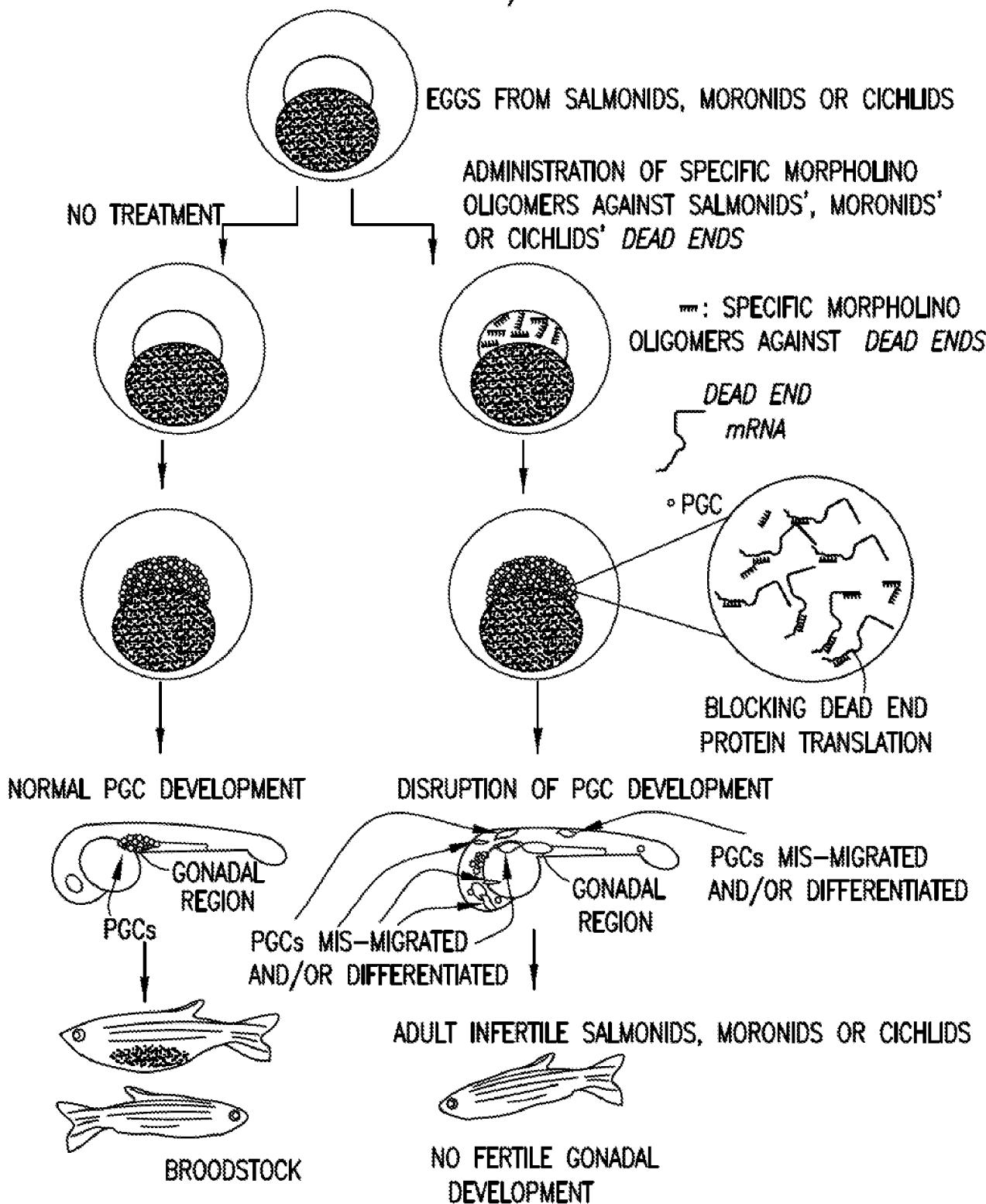


FIG.2

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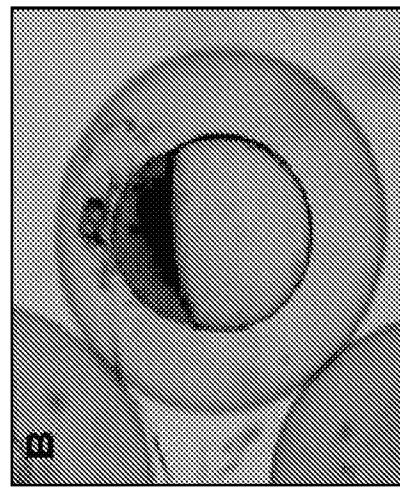


FIG.3B

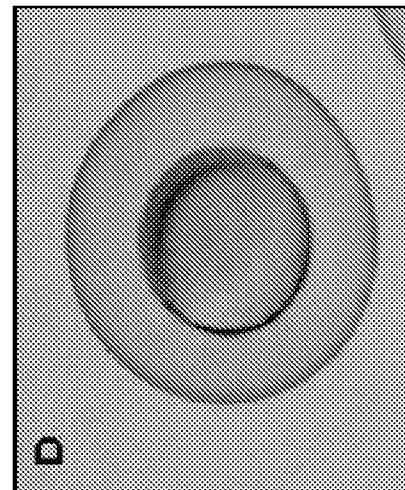


FIG.3D

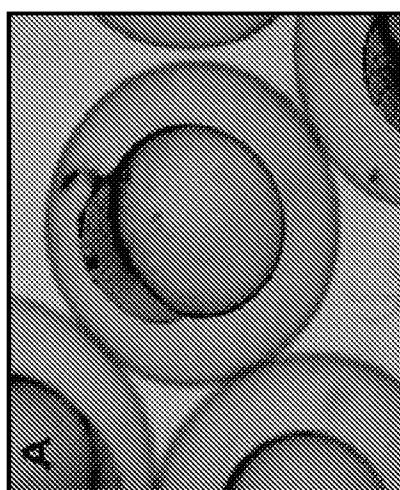


FIG.3A

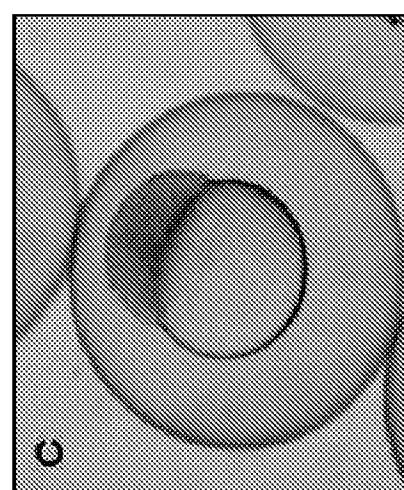


FIG.3C

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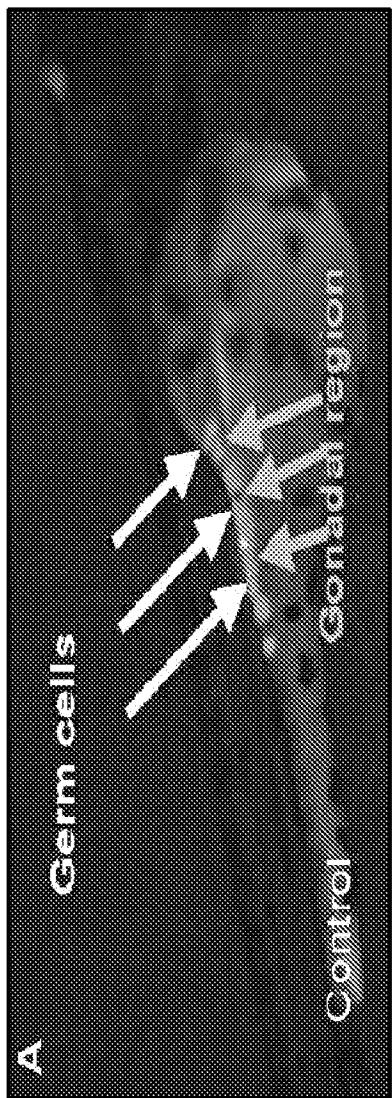


FIG. 4A

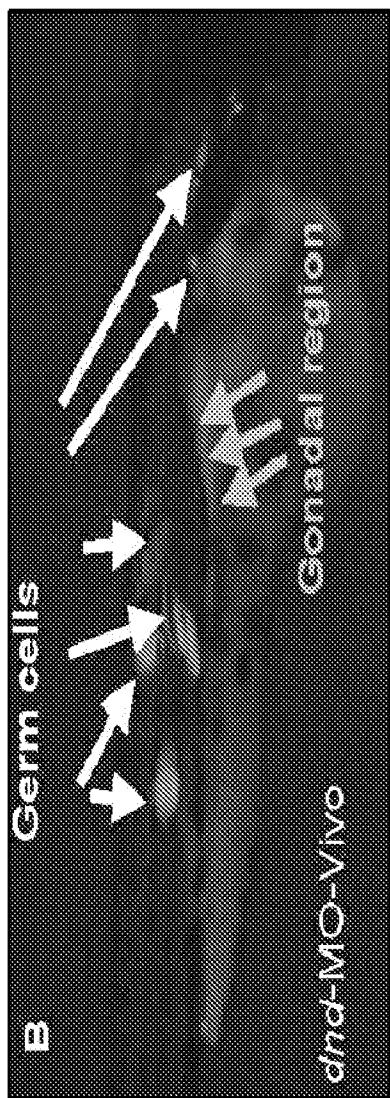
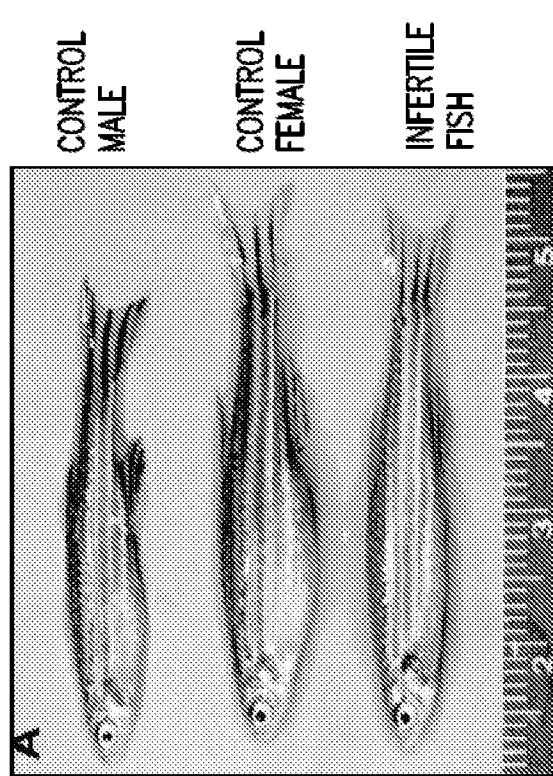
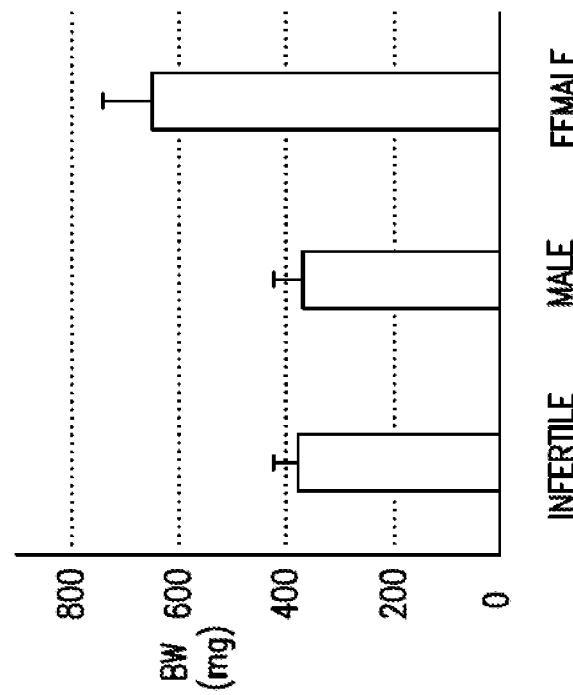


FIG. 4B

**FIG. 5A****FIG. 5B**

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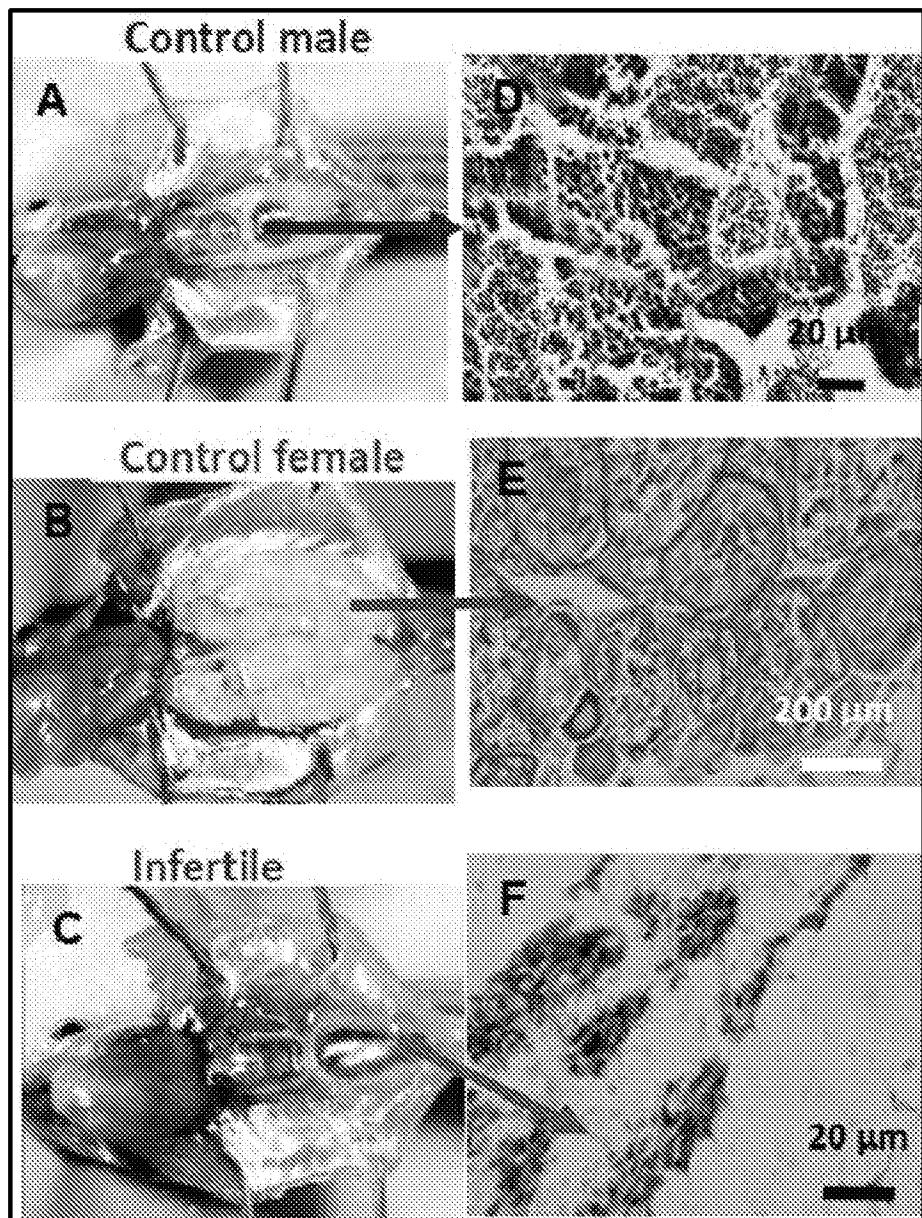


FIG.6

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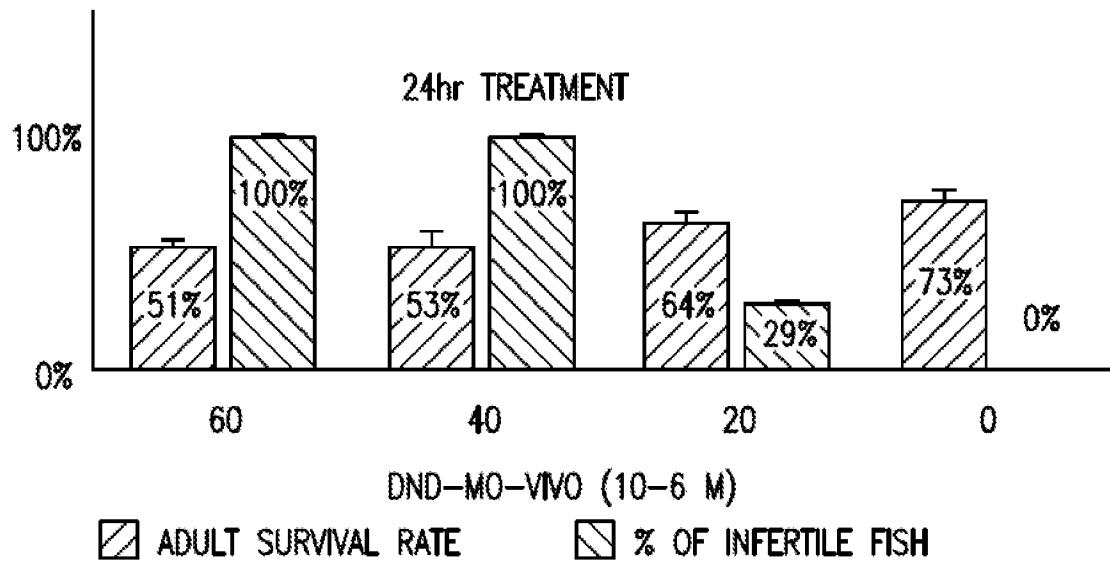


FIG.7A

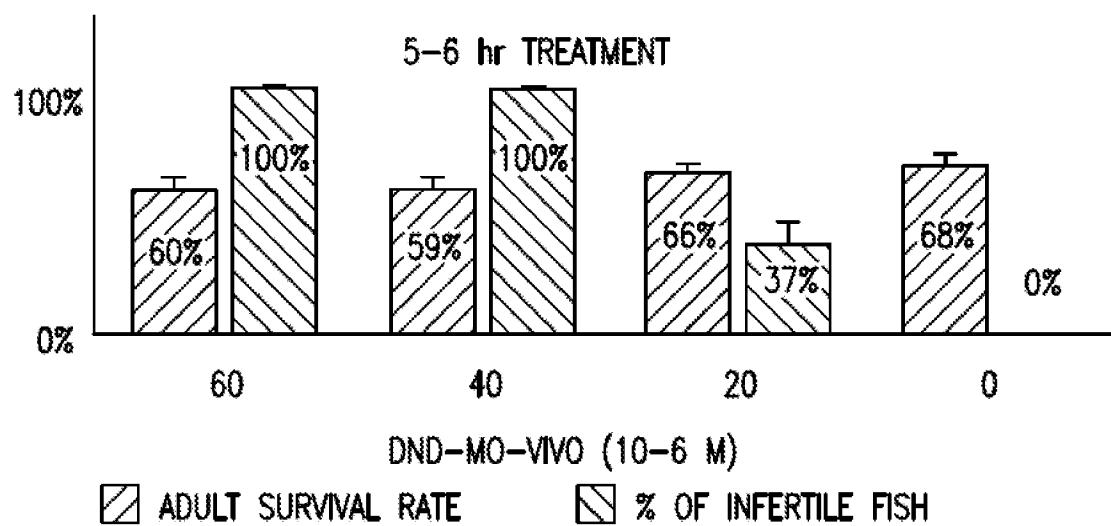


FIG.7B

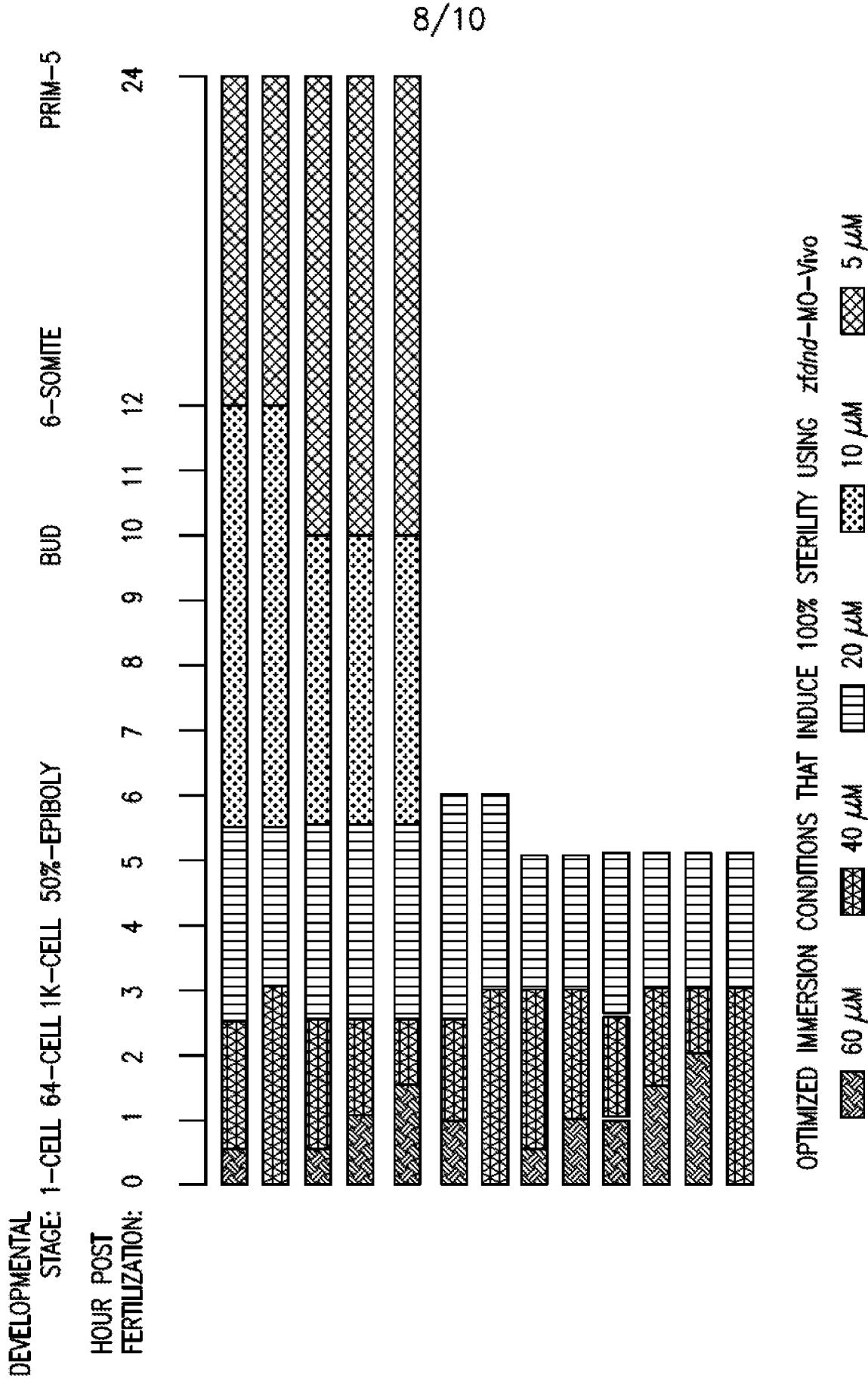


FIG.8

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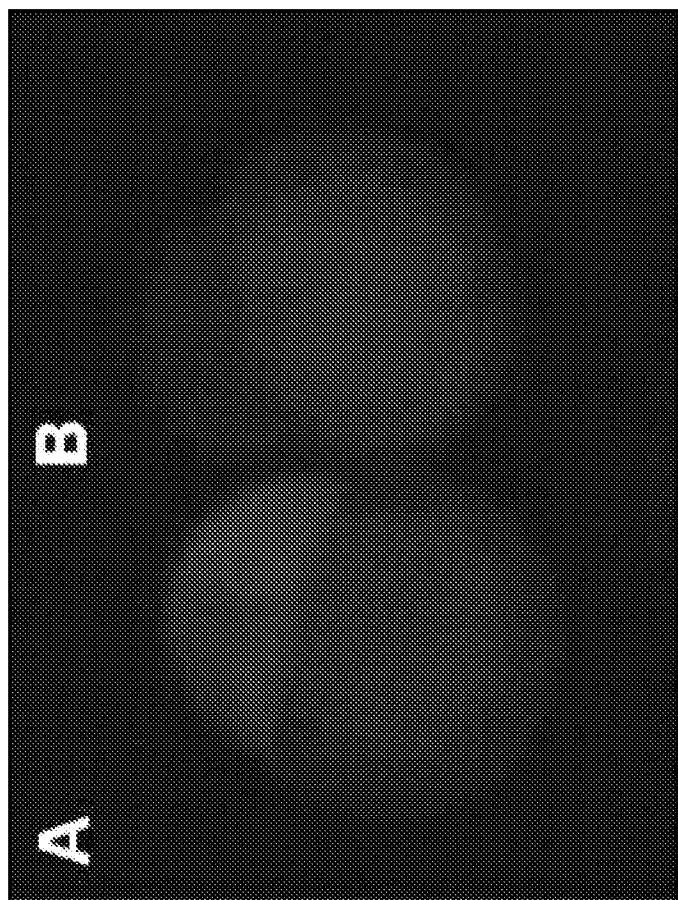


FIG.9

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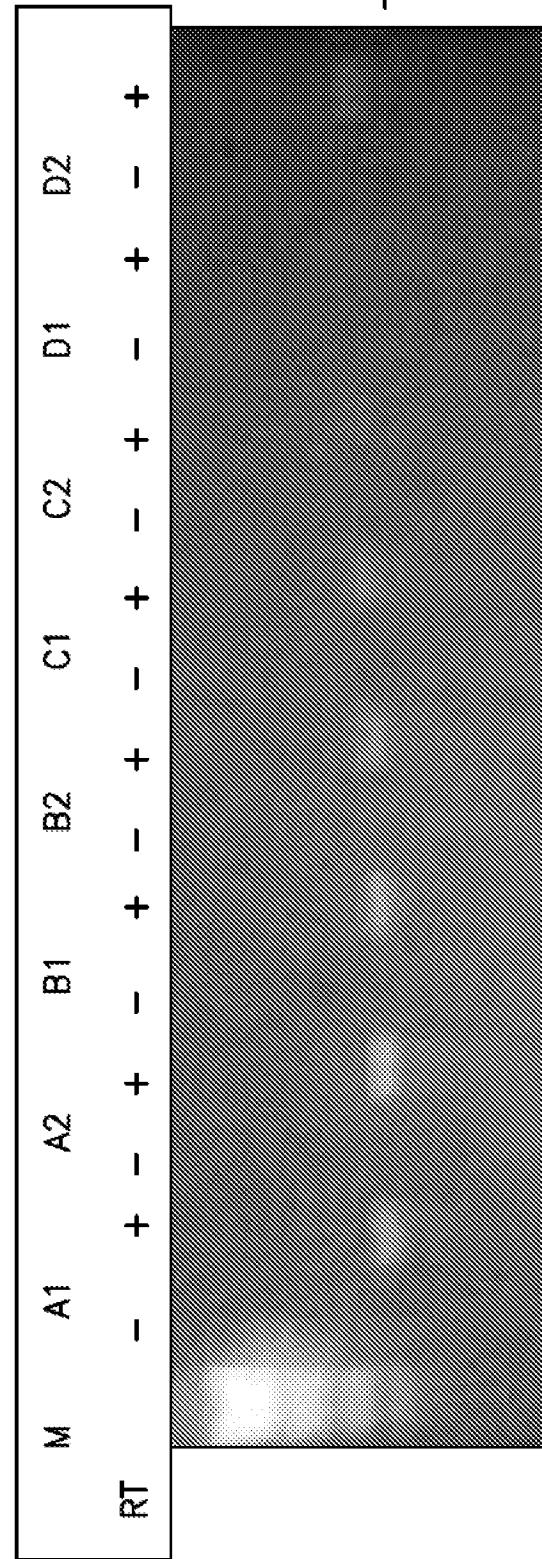
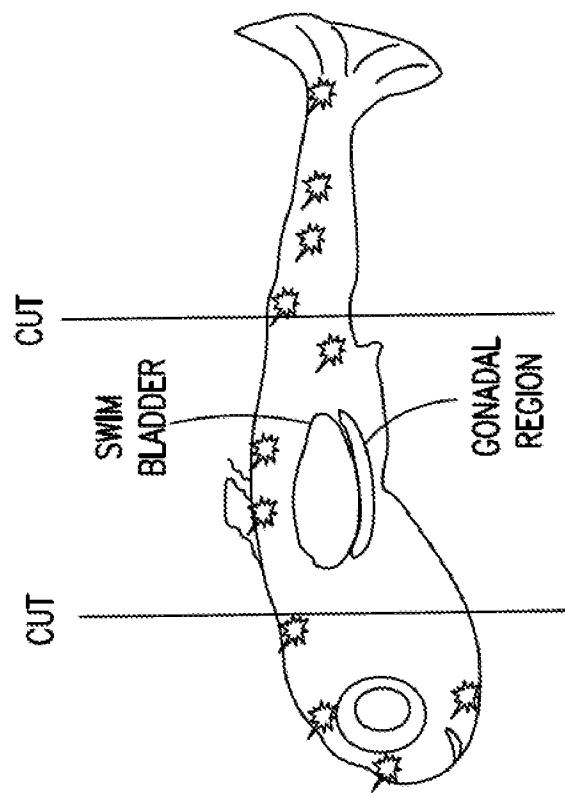


FIG. 10

SEQUENCE LISTING

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