

WHAT IS CLAIMED IS:

1. A method of generating a sterile sex-determined fish, crustacean, or mollusk, comprising the steps of:

breeding (i) a fertile ~~hemizygous~~heterozygous mutated female fish, crustacean, or mollusk having at least a first mutation and a second mutation with (ii) a fertile ~~hemizygous~~heterozygous mutated male fish, crustacean, or mollusk having at least the first mutation and the second mutation; and

selecting a ~~progenitor~~progeny that is homozygous by genotypic selection, the homozygous mutated ~~progenitor~~progeny being the sterile sex-determined fish, crustacean, or mollusk,

wherein the first mutation disrupts one or more genes that specify sexual differentiation and comprises: (a) a mutation in one or more genes that modulate the synthesis of androgen and/or estrogen; and/or (b) a mutation in one or more genes that modulate the expression of an aromatase Cyp19a1a inhibitor, and

wherein the second mutation disrupts one or more genes that specify gamete function and comprises: (c) a mutation in one or more genes that modulate spermiogenesis; and/or (d) a mutation in one or more genes that modulate: (aa) oogenesis; (bb) folliculogenesis; and/or (cc) a combination thereof.

2. A method of generating a sterile sex-determined fish, crustacean, or mollusk, comprising the step of:

breeding (i) a fertile homozygous mutated female fish, crustacean, or mollusk having at least a first mutation and a second mutation with (ii) a fertile homozygous mutated male fish, crustacean, or mollusk having at least the first mutation and the second mutation to produce the sterile sex-determined fish, crustacean, or mollusk,

wherein the first mutation disrupts one or more genes that specify sexual differentiation and comprises: (a) a mutation in one or more genes that modulate the synthesis of androgen and/or estrogen; and/or (b) a mutation in one or more genes that modulate the expression of an aromatase Cyp19a1a inhibitor,

wherein the second mutation disrupts one or more genes that specify gamete function and comprises: (c) a mutation in one or more genes that modulate spermiogenesis; and/or (d) a mutation in one or more genes that modulate: (aa) oogenesis; (bb) folliculogenesis; and/or (cc) a combination thereof, and

wherein the fertility of the fertile homozygous female fish, crustacean, or mollusk and the fertile homozygous mutated male fish, crustacean, or mollusk has been rescued.

3. The method of claim 2, wherein the fertility rescue comprises germline stem cell transplantation, and optionally sex steroid alteration, for example, an alteration of estrogen, or an alteration of an aromatase inhibitor.

4. The method of claim 3, wherein the germline stem cell transplantation comprises the steps of:

obtaining a-one or more germline stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the first mutation and the second mutation or a-one or more germline stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the first mutation and the second mutation; and

transplanting the one or more germline stem cells into a germ cell-less recipient male fish, crustacean, or mollusk, or into a germ cell-less recipient female fish, crustacean, or mollusk,

or

obtaining a-one or more spermatogonial stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the first mutation and the second mutation or a-one or more oogonial stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the first mutation and the second mutation; and

transplanting the one or more spermatogonial stem cells ~~into a testis of a germ cell-less fertile male fish, crustacean, or mollusk~~ or the one or more oogonial stem cells into an ovary of a germ cell-less fertile female fish, crustacean, or mollusk or a testis of a germ cell-less fertile male fish, crustacean, or mollusk.

5. The method of claim 4, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish, crustacean, or mollusk are homozygous for a null or loss of function mutation of the *dnd1*, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

6. The method of claim 4, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish, crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

7. The method of claim 6, wherein the germ cell-less fertile male fish, crustacean, or mollusk and the germ cell-less fertile female fish, crustacean, or mollusk are homozygous for the mutation of the *dnd*1, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

8. The method of claim 6, wherein the germ cell-less male fish, crustacean, or mollusk and the germ cell-less recipient female fish crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

9. The method of any one of claims 1-8, wherein the sterile sex-determined sterile fish, crustacean, or mollusk is a sterile male fish, crustacean, or mollusk.

10. The method of any one of claims 1-9, wherein the ~~first mutation comprises a mutation in~~ one or more genes that modulates the synthesis of androgen and/or estrogen- is:

~~11. The method of claim 10, wherein the first mutation comprises a mutation in~~ one or more genes that modulate the expression of: ~~(a)~~ aromatase Cyp19a1a, for example, one or more genes selected from the group consisting of *cyp19a1a*, *FoxL2*, and an ortholog thereof; ~~(b)~~ Cyp17, for example, *cyp17l* or an ortholog thereof; or ~~(c)~~ a combination thereof.

~~12~~11. The method of any one of claims 1-~~11~~10, wherein the ~~second mutation comprises a mutation in~~ one or more genes that modulate spermiogenesis- is:

~~13. The method of claim 12, wherein the second mutation comprises a mutation in~~ one or more genes that cause globozoospermia, for example, one or more genes that cause sperm with round-headed, round nucleus, disorganized midpiece, partially coiled tails, or a combination thereof, such as *Gopc*, *Hiat1*, *Tjp1a*, *Smap2*, *Csnk2a2*, and an ortholog thereof.

~~14~~12. The method of any one of claims 1-8, wherein the sterile sex-determined sterile fish, crustacean, or mollusk is a sterile female fish, crustacean, or mollusk.

~~15~~13. The method of any one of claims 1-8 and ~~14~~12, wherein the ~~first mutation comprises a mutation in~~ one or more genes that modulate the expression of ~~an~~ the aromatase Cyp19a1a inhibitor, ~~for example, is~~ one or more genes selected from the group consisting of *Gsdf*, *dmrt1*, *Amh*, *Amhr*, and an ortholog thereof.

~~1614~~. The method of any one of claims 1-8 and ~~1412-1513~~, wherein the ~~second mutation comprises a mutation in~~ one or more genes that modulate:

(a) oogenesis, ~~for example, is~~ one or more genes that modulate the synthesis of estrogen, such as FSHR or an ortholog thereof; ~~and/or~~

(b) folliculogenesis, ~~for example, is: (i)~~ one or more genes that modulate the expression of vitellogenins, such as *vtgs* or an ortholog thereof; or ~~(ii)~~ a mutation in a gene encoding or regulating: Vitellogenin; Estrogen receptor1; Cytochrome p450, family 1, subfamily a; zona pellucida glycoprotein; Choriogenin H; Peroxisome proliferator-activated receptor; Steroidogenic acute regulatory protein, or an ortholog thereof; ~~or~~

~~(c) a combination thereof.~~

~~1715~~. A method of generating a sterile sex-determined fish, crustacean, or mollusk, comprising the step of:

breeding (i) a fertile female fish, crustacean, or mollusk having one or more homozygous mutations with (ii) a fertile male fish, crustacean, or mollusk having one or more homozygous mutations to produce the sterile sex-determined fish, crustacean, or mollusk,

wherein the one or more mutations directly or indirectly disrupts spermiogenesis, and/or directly disrupts vitellogenesis, and

wherein the fertility of the fertile female fish, crustacean, or mollusk and the fertile male fish, crustacean, or mollusk have been rescued.

~~1816~~. The method of claim ~~1715~~, wherein the one or more mutations that directly or indirectly disrupts spermiogenesis is a mutation in *Gopc*, *Hiat1*, *Tjp1a*, *Smap2*, *Csnk2a2*, or an ortholog thereof.

~~1917~~. The method of claim ~~17-15~~ or ~~1816~~, wherein the one or more mutations that directly disrupts vitellogenesis is a mutation in a gene encoding or regulating: Vitellogenin; Estrogen receptor1; Cytochrome p450, family 1, subfamily a; zona pellucida glycoprotein; Choriogenin H; Peroxisome proliferator-activated receptor; Steroidogenic acute regulatory protein, or an ortholog thereof.

2018. The method of any one of claims 1715-1917, wherein the fertility rescue comprises germline stem cell transplantation, and optionally sex steroid alteration, for example, an alteration of estrogen, or an alteration of an aromatase inhibitor.

2119. The method of claim 2018, wherein the germline stem cell transplantation comprises the steps of:

obtaining a-one or more germline stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the homozygous mutation or a-one or more germline stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the homozygous mutation; and

transplanting the one or more germline stem cells into a germ cell-less recipient male fish, crustacean, or mollusk, or into a germ cell-less recipient female fish, crustacean, or mollusk.

2220. The method of claim 2119, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish, crustacean, or mollusk are homozygous for a null or loss of function mutation of the *dnd1*, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

2321. The method of claim 2119, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

2422. The method of any one of claims 1715-2321, wherein the fertile female fish, crustacean, or mollusk and the fertile male fish, crustacean, or mollusk have an additional homozygous mutation that specifies sexual differentiation.

2523. The method of claim 2422, wherein the mutation that specifies sexual differentiation modulates the expression of: (a) aromatase Cyp19a1a; (b) Cyp17, for example, a mutation in *cyp17l* or an ortholog thereof; (c) an inhibitor to aromatase Cyp19a1a, for example, a mutation in *Gsdf*, *dmrt1*, *Amh*, *Amhr*, or an ortholog thereof; or (d) a combination thereof.

2624. The method of any one of claims 1715-2323, wherein the breeding step comprises hybridization or hormonal manipulation and breeding strategies, to specify sexual differentiation.

2725. A fertile homozygous mutated fish, crustacean, or mollusk for producing a sterile sex-determined fish, crustacean, or mollusk, the fertile homozygous mutated fish, crustacean, or mollusk having at least a first mutation and a second mutation, wherein the first mutation disrupts one or more genes that specify sexual differentiation and comprises: (a) a mutation in one or more genes that modulate the synthesis of androgen and/or estrogen; and/or (b) a mutation in one or more genes that modulate the expression of an aromatase Cyp19a1a inhibitor, wherein the second mutation disrupts one or more genes that specify gamete function and comprises: (c) a mutation in one or more genes that modulate spermiogenesis; and/or (d) a mutation in one or more genes that modulate: (aa) oogenesis; (bb) folliculogenesis; and/or (cc) a combination thereof, and wherein the fertility of the fertile homozygous mutated fish, crustacean, or mollusk has been rescued.

2826. The fertile homozygous mutated fish, crustacean, or mollusk of claim 2725, wherein the fertility rescue comprises germline stem cell transplantation, and optionally sex steroid alteration, for example, an alteration of estrogen, or an alteration of an aromatase inhibitor.

2927. The fertile homozygous mutated fish, crustacean, or mollusk of claim 2826, wherein the germline stem cell transplantation comprises the steps of:

obtaining a-one or more germline stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the first mutation and the second mutation or a-one or more germline stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the first mutation and the second mutation; and

transplanting the one or more germline stem cells into a germ cell-less recipient male fish, crustacean, or mollusk, or into a germ cell-less recipient female fish, crustacean, or mollusk,

or

obtaining a-one or more spermatogonial stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the first mutation and the second mutation or a-one or more oogonial stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the first mutation and the second mutation; and

transplanting the one or more spermatogonial stem cells ~~into a testis of a germ cell-less fertile male fish, crustacean, or mollusk~~ or the one or more oogonial stem cells into an ovary of a germ cell-less fertile female fish, crustacean, or mollusk or a testis of a germ cell-less fertile male fish, crustacean, or mollusk.

~~3028~~. The fertile homozygous mutated fish, crustacean, or mollusk of claim ~~2927~~, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish, crustacean, or mollusk are homozygous for a null or loss of function mutation of the *dnd1*, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

~~3129~~. The fertile homozygous mutated fish, crustacean, or mollusk of claim ~~2927~~, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

~~3230~~. The fertile homozygous mutated fish, crustacean, or mollusk of claim ~~2927~~, wherein the germ cell-less fertile male fish, crustacean, or mollusk and the germ cell-less fertile female fish, crustacean, or mollusk are homozygous for the mutation of the *dnd1*, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

~~3331~~. The fertile homozygous mutated fish, crustacean, or mollusk of claim ~~2927~~, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

~~3432~~. The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-3331~~, wherein the sterile sex-determined sterile fish, crustacean, or mollusk is a sterile male fish, crustacean, or mollusk.

~~3533~~. The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-3432~~, wherein the ~~first mutation comprises a mutation in~~ one or more genes that modulates the synthesis of androgen and/or estrogen. is:

~~36. —The fertile homozygous mutated fish, crustacean, or mollusk of claim 35, wherein the first mutation comprises a mutation in~~ one or more genes that modulate the expression of: ~~(a)~~ aromatase Cyp19a1a, for example, one or more genes selected from the group consisting of *cyp19a1a*, *FoxL2*, and an ortholog thereof; ~~(b)~~ Cyp17, for example, *cyp17l* or an ortholog thereof; or ~~(c)~~ a combination thereof.

~~3734.~~ The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-3633~~, wherein the ~~second mutation comprises a mutation in~~ one or more genes that modulate spermiogenesis; is:

~~38.~~ ~~The fertile homozygous mutated fish, crustacean, or mollusk of claim 37, wherein the second mutation comprises a mutation in~~ one or more genes that cause globozoospermia, for example, one or more genes that cause sperm with round-headed, round nucleus, disorganized midpiece, partially coiled tails, or a combination thereof, such as *Gopc*, *Hiat1*, *Tjp1a*, *Smap2*, *Csnk2a2*, and an ortholog thereof.

~~3935.~~ The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-3331~~, wherein the sterile sex-determined sterile fish, crustacean, or mollusk is a sterile female fish, crustacean, or mollusk.

~~4036.~~ The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-33-31~~ and ~~3935~~, wherein the ~~first mutation comprises a mutation in~~ one or more genes that modulate the expression of an aromatase Cyp19a1a inhibitor; for example, is one or more genes selected from the group consisting of *Gsdf*, *dmrt1*, *Amh*, *Amhr*, and an ortholog thereof.

~~4137.~~ The fertile homozygous mutated fish, crustacean, or mollusk of any one of claims ~~2725-33-31~~ and ~~3935-4036~~, wherein the ~~second mutation comprises a mutation in~~ one or more genes that modulate:

(a) oogenesis; for example, is one or more genes that modulate the synthesis of estrogen, such as FSHR or an ortholog thereof; and/or

(b) folliculogenesis; is; for example, (i) one or more genes that modulate the expression of vitellogenins, such as *vtgs* or an ortholog thereof; or ~~(ii)~~ a mutation in a gene encoding or regulating: Vitellogenin; Estrogen receptor1; Cytochrome p450, family 1, subfamily a; zona pellucida glycoprotein; Choriogenin H; Peroxisome proliferator-activated receptor; Steroidogenic acute regulatory protein, or an ortholog thereof; ~~or~~

~~(c) a combination.~~

~~4238.~~ A fertile fish, crustacean, or mollusk having a homozygous mutation for producing a sterile sex-determined fish, crustacean, or mollusk, wherein the mutation directly or indirectly

disrupts spermiogenesis, and/or directly disrupts vitellogenesis, and wherein the fertility of the fertile fish, crustacean, or mollusk has been rescued.

4339. The fertile fish, crustacean, or mollusk of claim **4238**, wherein the mutation that directly or indirectly disrupts spermiogenesis is a mutation in *Gopc*, *Hiat1*, *Tjp1a*, *Smap2*, *Csnk2a2*, or an ortholog thereof.

4440. The fertile fish, crustacean, or mollusk of claim **4238** or **4339**, wherein the mutation that directly disrupts vitellogenesis is a mutation in a gene encoding or regulating: Vitellogenin; Estrogen receptor1; Cytochrome p450, family 1, subfamily a; zona pellucida glycoprotein; Choriogenin H; Peroxisome proliferator-activated receptor; Steroidogenic acute regulatory protein, or an ortholog thereof.

4541. The fertile fish, crustacean, or mollusk of any one of claims **4238-4440**, wherein the fertile fish, crustacean, or mollusk has a plurality of homozygous mutations that, in combination: directly or indirectly disrupt spermiogenesis; directly disrupt vitellogenesis; or both.

4642. The fertile fish, crustacean, or mollusk of any one of claims **4238-4541**, wherein the fertility rescue comprises germline stem cell transplantation, and optionally sex steroid alteration, for example, an alteration of estrogen, or an alteration of an aromatase inhibitor.

4743. The fertile fish, crustacean, or mollusk of claim **4642**, wherein the germline stem cell transplantation comprises the steps of:

obtaining **a-one or more** germline stem cells from a sterile homozygous male fish, crustacean, or mollusk having at least the homozygous mutation or **a-one or more** germline stem cells from a sterile homozygous female fish, crustacean, or mollusk having at least the homozygous mutation; and

transplanting the **one or more** germline stem cells into a germ cell-less recipient male fish, crustacean, or mollusk, or into a germ cell-less recipient female fish, crustacean, or mollusk.

4844. The fertile fish, crustacean, or mollusk of claim **4743**, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish,

crustacean, or mollusk are homozygous for a null or loss of function mutation of the *dnd1*, *Elavl2*, *vasa*, *nanos3*, or *piwi-like* gene.

4945. The fertile fish, crustacean, or mollusk of claim **4743**, wherein the germ cell-less recipient male fish, crustacean, or mollusk and the germ cell-less recipient female fish crustacean, or mollusk are created using ploidy manipulation, hybridization, or exposure to high levels of sex hormones.

5046. The fertile fish, crustacean, or mollusk of any one of claims **4238-4945**, wherein the fertile fish, crustacean, or mollusk has an additional homozygous mutation that specifies sexual differentiation.

5147. The fertile fish, crustacean, or mollusk of claim **5046**, wherein the mutation that specifies sexual differentiation modulates the expression of: (a) aromatase Cyp19a1a, for example, one or more genes selected from the group consisting of *cyp19a1a*, *FoxL2*, and an ortholog thereof; (b) Cyp17, for example, *Gsdf*, *dmrt1*, *Amh*, *Amhr*, or an ortholog thereof; (c) an inhibitor to aromatase Cyp19a1a; or (d) a combination thereof.

5248. The fertile fish, crustacean, or mollusk of any one of claims **4238-5147**, wherein producing a sterile sex-determined fish, crustacean, or mollusk comprises a breeding step comprising hybridization or hormonal manipulation and breeding strategies, to specify sexual differentiation.

5349. A method of making a fertile homozygous mutated fish, crustacean, or mollusk that generates a sterile sex-determined fish, crustacean, or mollusk, comprising the steps of:

breeding (i) a fertile hemizygous-heterozygous mutated female fish, crustacean, or mollusk having at least a first mutation and a second mutation with (ii) a fertile hemizygous heterozygous mutated male fish, crustacean, or mollusk having at least the first mutation and the second mutation;

selecting a progenitor progeny that is homozygous by genotypic selection; and
rescuing the fertility of the homozygous progenitor progeny,

wherein the first mutation disrupts one or more genes that specify sexual differentiation
and comprises: (a) a mutation in one or more genes that modulate the synthesis of androgen

and/or estrogen; and/or (b) a mutation in one or more genes that modulate the expression of an aromatase Cyp19a1a inhibitor, and

wherein the second mutation disrupts one or more genes that specify gamete function and comprises: (c) a mutation in one or more genes that modulate spermiogenesis; and/or (d) a mutation in one or more genes that modulate: (aa) oogenesis; (bb) folliculogenesis; and/or (cc) a combination thereof.