AQUAEXCEL

Aquaculture Infrastructures for Excellence in European Fish Research

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Deliverable D9.2

Optimization of androgenesis in carp and seabass

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Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
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Glossary

AQUAEXCEL: Aquaculture Infrastructures for Excellence in European Fish Research

mtDNA: mitochondrial DNA

Definitions

Androgenesis: a form of induced parthenogenesis in which the nuclear DNA of the organism is of paternal origin only. The unfertilised egg is irradiated to inactivate the maternal nuclear genome. Diploidy may be restored by suppression of mitosis in the haploid zygote (leading to dihaploid, completely homozygous). Note that the mitochondrial DNA (mtDNA) in an androgenetic individual will still be of maternal origin (the sperm does not pass on mtDNA to the zygote; egg mtDNA appears to be unaffected by radiation used to inactivate the maternal nuclear genome).

Gynogenesis: a form of induced parthenogenesis in which the nuclear DNA of the organism is of maternal origin only. The sperm is irradiated before fertilising the egg, to inactivate the paternal nuclear genome. Diploidy may be restored by suppression of the second meiotic division (leading to "meiotic" gynogenetic, partially heterozygous due to recombination at meiosis) or of mitosis in the haploid zygote (leading to "mitotic" gynogenetic, dihaploid, completely homozygous).

Homozygous clone founder: a completely homozygous individual produced by androgenesis or mitotic gynogenesis from an outbred clone founder. Genetically identical progeny produced from such an individual (by androgenesis or gynogenesis; in subsequent generations, hormonal sex reversal may be used to produce individuals of both sexes to allow propagation by normal crosses) will constitute an isogenic line.

Isogenic line: a group of genetically identical, completely homozygous individuals. Also called **fully inbred clonal line**. In this context, homozygous clone founders are produced by induced androgenesis or mitotic gynogenesis from outbred clone founders, then these founders are propagated by androgenesis or gynogenesis. In subsequent generations, hormonal sex reversal may be used to produce individuals of both sexes to allow propagation by normal crosses.

Outbred clone founder: an individual used as a source of eggs or sperm to produce a homozygous clone founder by androgenesis or mitotic gynogenesis. Such an individual may come from an outbred population, a defined strain or a line selected for particular traits. Such individuals would be expected to be heterozygous at a proportion of loci.

Appendix 1 illustrates the production of an isogenic line using androgenesis.

Summary

Objectives

The objective of this deliverable was to optimize androgenesis as a technique for developing isogenic lines in European seabass and common carp.

Both androgenesis and mitotic gynogenesis have been induced in several fish species. Androgenesis is inherently more challenging (largely due to the greater difficulties of successfully inactivating the maternal nuclear genome in the unfertilised egg), but attractive in species where males mature earlier than females (fairly common in fish) as a means to shorten the time taken to produce isogenic lines. In both techniques, inactivation of one parental genome produces a haploid zygote; diploidy is restored by giving a pressure or temperature shock to suppress mitosis, resulting in a "dihaploid" embryo (two identical sets of chromosomes, thus completely homozygous).

The term "optimize" should be qualified here. Yields of androgenetic or mitotic gynogenetic individuals (as survivors at first feeding stage) are normally low – in general a few percent relative to that observed in control groups (untreated sperm and eggs, normal crosses) – and variable. Some of this variation is due to egg quality (poor egg quality will give disproportionately low yields of androgenetics or mitotic gynogenetics, often zero) and the genetics of the individuals used as outbred clone founders (e.g. recessive lethal or deleterious alleles). In practice, the objective is to be able to produce enough homozygous clone founders to then be able to go on to produce isogenic lines. Homozygous clone founders are completely inbred, so an (unpredictable) proportion will have reduced reproductive capacity.

Rationale

Androgenesis and mitotic gynogenesis have been induced before in common carp (reviewed by Komen and Thorgaard, 2007). The approach pursued by VURH and HAKI on common carp was thus verification and comparison of methodology based on published methods. In particular, the use of ultraviolet (UV) irradiation and gamma (γ) irradiation for inactivation of the female (egg) nuclear genomes was compared, while parallel induction of androgenesis and mitotic gynogenesis allowed some comparison of these methods. γ irradiation has greater penetrance than UV, making it in theory easier to treat eggs, but γ sources are only available in specialised laboratories, while the equipment for UV irradiation is widely available.

In sea bass, methodology for mitotic gynogenesis has been published, but not for androgenesis. The approach pursued at Palavas was to test methodology for inducing androgenesis in sea bass using UV irradiation (based on published methodology on mitotic gynogenesis in sea bass and androgenesis in other species), while producing mitotic gynogenetic sea bass in parallel for comparison and as an alternative route to isogenic lines.

Main findings

Androgenesis was successfully induced in common carp using both methods (UV and γ irradiation of eggs), but the survival rate using γ irradiation was much lower than with UV. This was primarily due to the logistics of using γ irradiation – stripped eggs have to be chilled, taken to the γ source, brought back to the lab and warmed up again before continuing with fertilization, heat shock and incubation. In contrast, UV irradiation was carried out in the lab in VURH, with minimal delay between stripping and fertilization.

Putative common carp homozygous clone founders (androgenetic and gynogenetic) were tested using scaling pattern markers (some strains) and microsatellite markers to distinguish androgenetics or gynogenetics from biparental diploids (irradiation failures) or meiotic gynogenetics (gynogenesis only). Mitotic gynogenetic carp were successfully produced in both VURH and HAKI, in higher numbers than androgenetics. Both are being grown on to maturity to produce isogenic lines.

Despite exhaustive testing of UV irradiation, no haploid androgenetic sea bass embryos were produced at IFREMER. Sea bass eggs are fairly small and transparent, so should be suitable for successful UV irradiation. Mitotic gynogenesis carried out over the same spawning season produced survivors which are being grown on as putative homozygous clone founders (will be analysed with genetic markers when large enough to tag).

Teams involved

Common carp: VURH, HAKI, INRA, UoS
Sea bass: IFREMER, INRA, Ugent, UoS
UoS is the author of this deliverable.

Geographical areas covered

Czech Republic, Hungary, France, UK; isogenic lines are intended to be made available more broadly.

1. Androgenesis in common carp

1.1. VURH: Androgenesis using UV irradiation

1.1.1. Irradiation of eggs

The first series of preliminary experiments at VURH were carried out to check different media for keeping common carp ova during UV-irradiation and to verify the best UV-irradiation dose for androgenesis, based upon the colour of progeny using eggs of common carp (wild colour, dominant) and sperm of koi carp (yellow colour, recessive). We also tested three different media for egg storage - carp artificial fluid (CAF) according to Bongers et al. (1994); common physiological saline and/or Ringer´s solution. Initial experiments showed that Ringer's was the best, while with CAF ova remained stuck on the glass and they all died.

Preliminary testing of optimal irradiation was based on irradiation of wild type (dominant green) common carp egg and fertilization with sperm of koi carp (recessive) where fertilization of irradiated eggs resulted in yellow colour (koi) haploid progeny. A dose of 600 000 μJ.cm^{-2} was shown to be optimum according to wild type /koi progeny in individual treatments.

1.1.2. Large scale androgenesis

Mass androgenesis was performed with 300 g of eggs from each female which were irradiated (after mixing 1:2 in Ringer's solution) using a UV Crosslinker with 600 000 μJ.cm-2 dose (3x100g of eggs irradiated on glass slides 25x27 cm). Eggs were placed on glass slides covered by an agarose layer to eliminate the egg sticking during irradiation. Irradiated eggs of individual females were immediately fertilized by adding 2 ml of untreated sperm of individual males with 150 ml hatchery water at 24°C. After two min, activated eggs were placed into a container with mesh bottom and incubated at 24°C. At 30 min after gamete activation, a heat shock at 40°C (in milk solution) with 2 min exposure was given to restore diploidy. The eggs were then transferred back to 24°C milk solution. All temperature treatments were realized in 40l tanks. Total time of egg desticking was 1 h (in common carp, it is necessary to treat the eggs, e.g. with milk solution, to prevent them from sticking to each other during incubation, which can lead to egg mortality). Eggs were then repeatedly rinsed with hatchery water and put into Zuger jars for incubation.

Irradiated eggs of scaly Ropsha carp (scaly type, genotype SSnn) were fertilized by sperm of M72 males (mirror type carp, genotype ssnn) to allow control of irradiation success and androgenesis by scaling. This type of control is not possible for Ropsha carp androgenesis. After initial problems with generation of androgenetic progeny and aquaria rearing we generated about 160 androgenetic juveniles of M72 and 200 androgenetic juveniles of Ropsha carp (size about 5 cm) in 2012.

1.2. HAKI: Androgenesis using gamma irradiation

1.2.1. Irradiation of eggs

Androgenesis was performed in HAKI based on a completely different way of inactivation of genome of eggs, where 60Co isotope was used for irradiation. Based on Bercsényi et al. (1998) and Bongers et al. (1994) where irradiation dosage, shocking time and temperature were extensively described, androgenesis induction in HAKI was carried out following standard, optimized values for these parameters. Optimization of two other not well described parameters was carried out according to two approaches. In case of androgenesis the quantity of the irradiated eggs is a limiting factor. In case of UV only a thin layer of eggs can be irradiated and the uniformity of the absorbed radiation is not perfect. In order to increase the quantity of irradiated eggs and the uniformity of absorbed radiation we used γ ray. Since this type of ray source is not a portable device, and is 1 hour drive from the fish farm, the eggs to be irradiated were transported at 0-2oC from the farm to the site of irradiation. The question here was: how can we uniformly cool down the eggs after stripping and how their warming up before fertilization affects the rate of androgenic fry. We found that best results were achieved when cooling of eggs after ovulation was carried out in a plastic bag (600-800 g of eggs) where the bag was pressed as a 10-15 mm thick quilt and laid on melting ice. This provided a ca. 10% increase of the fertilization rate in comparison to the “in bucket” cooled eggs at the fertilization 1.5 hours after the ovulation.

We also studied how the speed of warming up of eggs (after cooling as described above) affected the fertilization rate. We found no significant effect of the duration of warming up to 22oC, the temperature of fertilization – between practical limits (0-15 minutes). After a longer warming up period the fertilization rate drastically decreased, both in irradiated and in control eggs.

1.2.2. Production of diploid androgenetics

After subjecting eggs to γ-irradiation as described above, eggs were fertilized with untreated milt. 30 min after gamete activation, a heat shock at 40°C water with 1 min exposure was given to restore diploidy (Nagy et al., 1978; Bercsényi et al., 1998).

Because of low survival and high sensitivity of several hundreds of generated progeny (see Table 1) we obtained only 35 individuals suitable for genotyping, from which 2 androgenetic juveniles of S15 line and 2 androgenetic juveniles of Amur carp (size about 5 cm) were proved to be fully homozygous in the report period. The following markers were used for the analysis: HLJ2175, HLJ2295, HLJ2346, HLJ2371, HLJ2465, HLJ2496, HLJ2544, HLJ2571, HLJ334, HLJ380, HLJ526, HLJ534, HLJE265 (from the linkage map of Zhang et al 2011). These were selected as a suite of largely telomeric markers for screening putative androgenetic and gynogenetic fish (capable of distinguishing between biparental and uniparental individuals, and between mitotic and meiotic gynogenetics, depending on parental genotypes).

Table 1: Survival of androgenetic and gynogenetic carp produced in HAKI in the 2011 breeding season.

Group	Fish to Aquaria	Number of fish	Fish to RAS	Number of fish	Total weight (g)	Fish to pond	Number of fish	Total weight (g)
Gyno Amur	23/05/2011	2000	17/06/2011	1200	478	21/06/2012	370	51817
Gyno 15	23/05/2011	430	17/06/2011	100	96	21/06/2012	46	15966
Andro Amur	28/05/2011	29	17/06/2011	19	20.2	21/06/2012	8	5541
Andro 15	28/05/2011	63	17/06/2011	25	30.6	21/06/2012	14	7296

RAS = recirculating system; Gyno = putative gynogenetics; Andro = putative androgenetics; Amur = Amur strain; 15 = Szarvas S15 strain

2. Androgenesis in European sea bass

Methodology for induced mitotic gynogenesis in sea bass has been published (Francescon et al., 2004), but not for androgenesis, the main objective of this part of Deliverable 9.2. The base populations originated from Ifremer experimental populations and lines: wild West-Mediterranean and wild Atlantic origin and also lines/families selected for different traits. Since the species is characterized by a polygenic sex determinism and no sex chromosomes, it is expected that isogenic lines will contain both males and females therefore no sex-reversal treatments will be performed.

Since no haploid androgenetic larvae were produced from the trials to optimise UV irradiation of eggs (see below), diploid mitotic gynogenesis was also induced (using established protocols), as the alternative way of developing isogenic lines.

2.1. Summary of realized operations

- November 2011 (M9): recruitment of a PhD student, Julie COLLETER based in Palavas-les-Flots, France in the Ifremer team belonging to the UMR 110 " INTensification Raisonnée et Ecologique Pour une PIsciculture Durable, INTREPID".
- November-December 2011 (M9-M10): training of the PhD student at University of Stirling on tilapia androgenesis and gynogenesis.
- January 2012 (M11): setting up of a working team, and irradiation and pressure shock devices.
- February to April (M12-M14): optimization of sea bass eggs UV treatment to

produce the first mitotic androgenetic offsprings.

- April-May (M14-M15): production of the first mitotic gynogenetic offsprings.
- Since May (M15-M17): larval rearing of the different gynogenetic offsprings.

2.2. Androgenesis

2.2.1. Materials and methods

To optimize the haploidy induction, the following protocol was used:

- Irradiation was provided by a set of 8 UV lamps (254 nm, 12W) fixed over and below a quartz plate (500x600 mm) gently stirred by an agitator, and equipped with 7 quartz Petri dishes (85 mm diameter each) receiving egg samples.
- 4 UV intensities were tested: 720, 1 320, 2 900 and 4 200 erg/min/mm² (7 200, 13 200, 28 800 and 42 000 µJ/cm²/min) covering a wide range found in the literature to successfully induce androgenesis in several species. For each tested UV intensity, a duration of irradiation comprised between 0 to 12 minutes was applied in triplicate (3 successive runs for the same pool of eggs). Each UV intensity was tested twice.
- Each test was done on egg pools stripped from 3 to 5 females fertilized post-irradiation by a pool of fresh sperm stripped from 1 to 3 males.
- Eggs and sperm were diluted previous irradiation with a home-made medium (C. Fauvel, pers. comm.) containing Stor-fish™ (IMV Technologies).
- Each sample of treated egg (3 ml) was incubated until hatching (≈60 degree.day; 4 days at 14.2°C) in an individual incubator (2 l each) smoothly homogenized with air and seawater (salinity 37-39ppt). The incubation device was composed of 40 individual incubators immerged in a raceway provided with recirculated seawater.
- Fertilization rate was evaluated 4-8 h post fertilization (PF) (2-4 degree.day at 14°C). It was evaluated by counting the number of eggs presenting 4 to 8 cells in a sample of about 200 eggs.
- Success of embryonic development is evaluated 50 h and 74 h PF (respectively 30 and 50 degree.days at 14°C). It is evaluated by counting the number of developing embryos in a sample of about 200 eggs.
- For each irradiation level, embryo malformations were checked and photographed, and larvae that had successfully hatched were sampled for flow-cytometry analysis to assess the ploidy status.

2.2.2. Results

During the 3 months of the natural reproduction season that were exploited, 75 females were used to test the different levels of UV irradiation with a selection of the best spawns to constitute the egg pools; a total of 192 trials were realized (4 doses x 6-7 durations, 3 runs for each combination).

The range of UV irradiation tested (comprised between 720 and 4 200 erg/min) never led to haploidy induction. Whatever the UV dose tested, the survival rate (relative to control) rapidly decreased from 100% to less than 10% when eggs were irradiated 1 to 6 min, and progressively reached 0% for an irradiation of 10-12 min (fig.1). Despite some larvae showed malformations (fig.2), which may suggest the “haploid syndrome”, all samples from hatched larvae analyzed using flow-cytometry were diploid. Some samples were suspected to be aneuploid but their quality was so bad that it was impossible to conclude on their ploidy status.

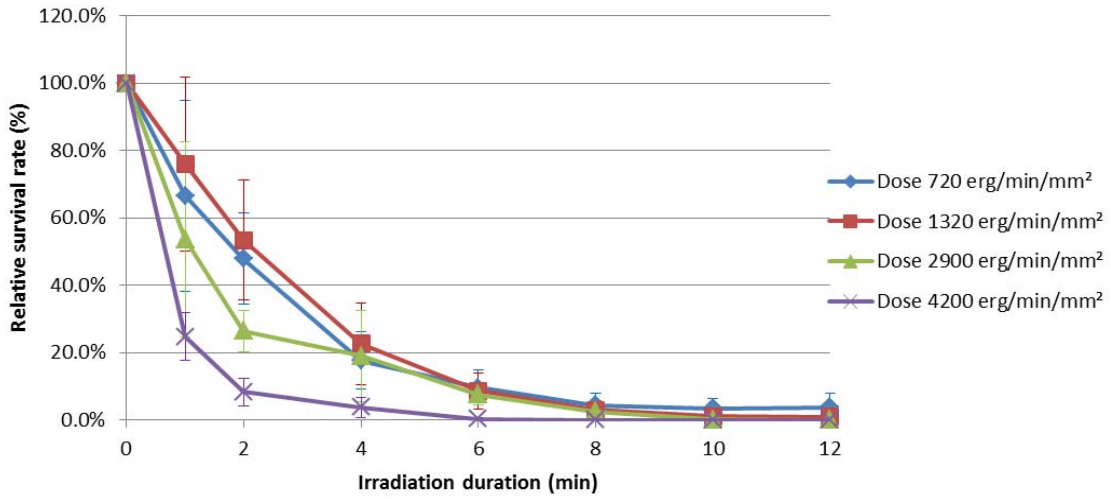


Fig.1. Haploidy induction in sea bass: evolution of the relative survival rate of eggs submitted to different durations and levels of UV irradiation. The UV range was 720 - 4 200 erg.min^{-1}.mm^{-2}; the duration range: 0 - 12 min. Relative survival rates were based on control fertilization rate and an average of the survival rates (embryonic development) estimated 50 h and 72 h post-fertilization (n=1200, n=200 per treatment). Means are surrounded by standard deviations. The control fertilization varied from 40 to 70%.

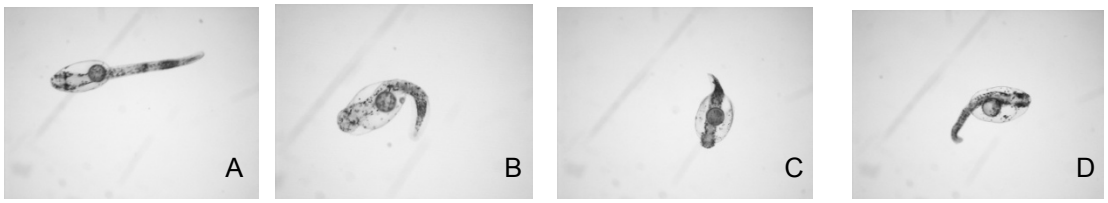


Fig. 2. Different types larvae obtained at hatching for a UV irradiation of 1320 erg.min^{-1} during 2 minutes. A: normal larva; B, C and D: malformed larvae.

2.2.3. Following steps

- Histological preparations of UV treated eggs to analyze the effect of UV (UVC: 254nm) on DNA and to describe the androgenesis process at a cellular level.
- Experiments to determine the UV absorption capacities of eggs.
- Karyotypes to analyze the effect of UV on chromosomes.

2.3. Gynogenesis

In the initial planning, it was foreseen to produce the clones through androgenesis because of the late maturation of the sea bass dams (precocious males are fluent at one-year-old). Since the process to obtain androgens is not yet mastered, several batches of mitotic gynogens were produced.

2.3.1. Materials and Methods

The following protocol was used to produce mitotic gynogenetic offsprings:

- Sperm stripped from 1 to 2 males, was diluted 1:20 (sperm vol: medium vol) with a home-made medium (C. Fauvel, pers. comm.) containing Stor-fish™ (IMV Technologies) previously to irradiation.
- Irradiation was provided by a set of 8 UV lamps (254 nm, 12W) fixed over and below a quartz plate (500x600 mm) gently stirred by an agitator, and equipped with 1 or 2 quartz Petri dishes (85 mm diameter each) receiving sperm samples. The chosen UV dose was 32 000 erg.mm^{-2} (32 0000 µJ/cm²) according to Peruzzi and Chatain (2000).
- 25 females were stripped and treated separately, only the good quality spawns with a consequent volume of eggs being used. The fish populations originated from a domesticated Atlantic line and artificially delayed (2 months) spawners.
- Each used breeder was fin-clipped, and the tissue sample stored in ethanol 100°. Each batch of eggs and sperm sample was marked and treated separately.
- Respective volumes of sperm and ovule used for each treated batch was 5 ml of diluted sperm for 150 ml of ovule (1:600 sperm vol: ovule vol), which corresponds to an unlimited availability of spermatozoids (1 500 times over the recommended ratio for a maximum fertilization rate according to Fauvel *et al.,* 1999).
- Just after fertilization, egg batches were stored at 14°C until the pressure shock was applied to reestablish the diploidy of the embryos. The pressure device was a hydraulic press (Enerpac). The shock timing was calculated using the equation of Francescon (2004) based on the first cleavage timing. The treatment was 8 500 psi for 4 minutes according to Peruzzi and Chatain (2000).
- A haploid control (no pressure shock) was performed for each batch of eggs, and the hatched larvae sampled for flow-cytometry analyzes.
- Eggs were incubated for 3 days in 40 l tanks at 14°C, and just before hatching transferred in larval rearing tanks (100 l each) where temperature was regulated at 16°C.
- Each tank was marked with the female number used to produce the offsprings.

2.3.2. Results

On the 25 batches of mitotic gynogenetic offsprings realized, 8 are currently on-growing in the larval rearing unit. They presently aged 2 to 3 months according to the batch, and represent all together, a potential batch of about 1 000 mitotic gynogens. To avoid killing any of them, and because the survival rate was very low, no biometry was carried out.

Flow-cytometry analysis executed on the haploid controls showed that all hatched larvae were haploid; they died within a few days after hatching. The figure 3 shows the result of a flow-cytometry analyze of haploid larva and a diploid control. Pictures taken at hatching in the haploid controls also clearly demonstrate the “haploid syndrome” as described in many other species: malformations, short body… (Fig.4).

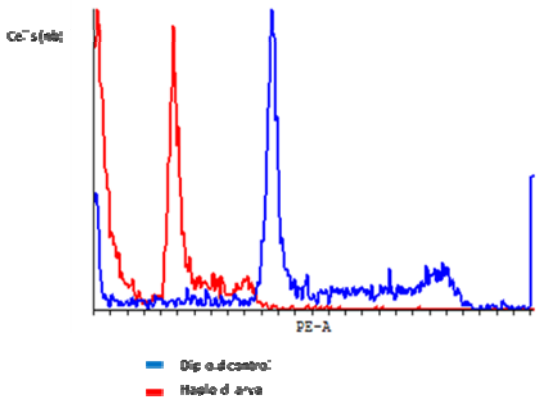


Fig. 3. Flow-cytometry analysis of haploid and diploid sea bass larvae. The blue curve shows a great peak which indicates that most of the cells are diploid, the small peak shows some tetraploid cells very usual in a growing larva where cell divisions occur very often. The red curve shows a great peak at approximately half the position of the blue one on the PE-A axis, which indicates that most of the cells are haploids.

Fig. 4. Different haploid larvae produced by mitotic gynogenesis clearly showing the “haploid syndrome”.

2.3.3. Following steps

- Growing of the potential mitotic gynogenetic offsprings until maturation.
- Assessment of the uniparental origin and homozygous status using microsatellite markers.
- Karyotypes and histological preparations to describe the gynogenesis process at a cellular level.

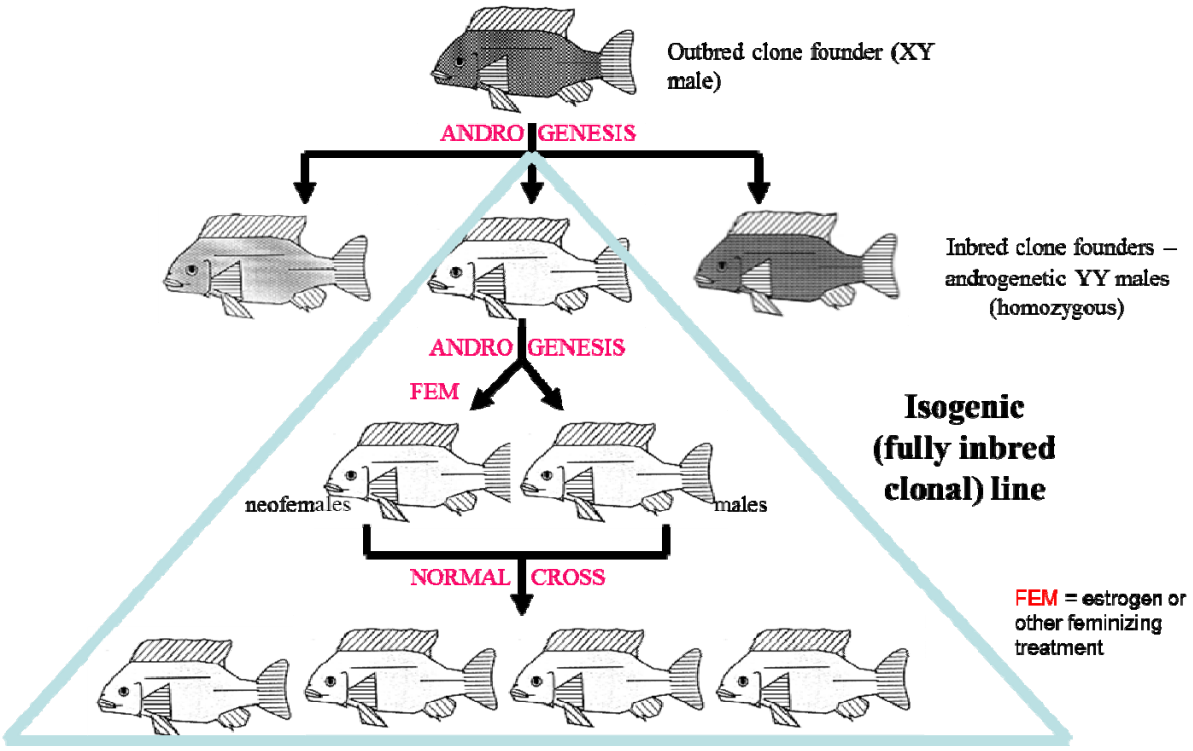
Conclusions

- Androgenesis was successfully induced in common carp using both UV and γ irradiation to inactivate the egg nuclear genome.
- Higher yields of androgenetic common carp were produced using UV irradiation than γ irradiation
- For UV irradiation, the numbers of androgenetic common carp produced appears to be large enough to develop isogenic lines
- Large numbers of mitotic gynogenetic common carp were also produced, and can also be used to develop isogenic lines but with a longer generation time than via androgenesis.
- UV irradiation of unfertilised sea bass eggs was not successful in producing haploid androgenetic embryos
- Large numbers of putative mitotic gynogenetic sea bass were also produced, and females from this can also be used to develop isogenic lines through gynogenesis but with a longer generation time than via androgenesis.

Appendix 1:

Example of development of an isogenic line, in an XX/XY species via androgenesis

The diagram below illustrated the development of a YY isogenic line in a species with XX/XY sex determination, using androgenesis (and hormonal feminization in later generations). A single outbred clone founder will generate inbred clone founders that differ from each other, reflecting heterozygosity in the outbred clone founder and recombination leading to gamete (sperm) production. Thus isogenic lines generated from sibling inbred clonal founders will vary from each other.



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Annex 1

Deliverable Check list (to be completed by Deliverable leader)

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