AQUAEXCEL

Aquaculture Infrastructures for Excellence in European Fish Research

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Combination of CP & CSA
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Capacities

Deliverable D9.3

Phenotypic analysis in G1 offspring in salmon and A1 offspring in seabass and carp

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Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (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 [A1] or mitotic gynogenesis [G1] from an outbred clone founder. Genetically identical progeny produced from such an individual (by androgenesis [A2] or gynogenesis [G2]; 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 mitotic gynogenesis or androgenesis. 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 for this deliverable was to analyse the phenotypes of G1 offspring in Atlantic salmon (*Salmo salar*) and A1 offspring in European seabass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*). G1 offspring are dihaploid mitotic gynogenetic offspring of outbred (female) founders, while A1 offspring are dihaploid androgenetic offspring of outbred (male) founders. These are also referred to as "clone founders" – since they are dihaploid (two completely identical sets of chromosomes), a further generation of gynogenesis or androgenesis results in isogenic clones. Successful production of G1 and/or A1 individuals is thus a vital step on the way to producing isogenic clonal lines.

After verification of putative A1 and G1 individuals using appropriate molecular markers (that can distinguish maternal and paternal contribution, and also can distinguish between mitotic and meiotic gynogenetics: multiple markers are required for this), in some cases phenotypic variation may be observed among groups of dihaploids. Such variation is a promising indicator of phenotypic variation among isogenic clonal lines that may be founded from such individuals (one of the major objectives of developing such isogenic lines). There is, however, a limit to the types of phenotypic assessment that can be carried out on A1 and G1 fish, given their importance and potential clone founders. For example, once a line is founded (A2, G2 and further generations) and multiple individuals are available, destructive testing for phenotypes such as disease resistance becomes possible. Another important consideration is the fertility of A1 and G1 individuals: dihaploids are completely inbred and it is thus expected that reproductive traits will be compromised in at least a moderate proportion of such individuals, meaning that isogenic clonal lines can only be founded from a proportion of A1 and G1 individuals, and that this can only be determined once these fish have matured.

Main findings

This report contains information on phenotypic variation in G1 Atlantic salmon plus a small number of G1 European seabass. The G1 nature of these fish has been analysed using multiple DNA markers. There is also information on the phenotypes of some fish produced during mitotic gynogenesis trials on European seabass that are concluded not to be G1 fish (i.e. heterozygous at some markers and/or paternal contribution detected).

The production of G1 fish in Atlantic salmon is the most advanced part of the development of clonal lines in workpackage 9. These fish exhibit wide variation in coloration (spotting pattern) and body shape (condition factor, K).

Two G1 sea bass have been verified by microsatellite markers. These are of normal appearance, while deformities were observed among fish that have some level of heterozygosity of maternal origin (presumed spontaneous meiotic gynogenetics) or have biparental contribution (UV presumed irradiation failure). However, the number of G1 fish is too small to draw any conclusions.

A1 fish have not been produced to date in European seabass due to lack of success in extensive trials to optimise UV irradiation in seabass eggs (to induce haploid androgenesis, the first step in developing androgenesis, which is then followed with a pressure or temperature shock to the fertilised eggs to induce diploidy by blocking mitosis). Because of this, production of G1 fish was initiated.

Putative A1 and G1 fish have been produced in common carp but problems have been experienced with survival and high proportions of non-homozygous fish. No data on

phenotypic variation in A1 or G1 fish is available. Analysis of the surviving juvenile fish using molecular markers allowed the identification of 99 A1 and 43 G1 individuals.

Trials have been carried out on X-ray androgenesis in salmon, as an alternative to UV (it is more difficult to apply UV irradiation evenly to fish eggs than to fish sperm). These look promising but dihaploids have not yet been produced.

Teams involved
Atlantic salmon: IMR, UoS
Sea bass: IFREMER, INRA, Ugent, UoS
Common carp: VURH, HAKI, INRA, UoS

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

1. Atlantic salmon

On 7th August 2012 the surviving G1 fish were PIT tagged and a small fin-clip was taken for genotyping. DNA was extracted from fin-clips. This was performed in 96-well format using a commercially available extraction kit (Qiagen DNeasy®96 Blood & Tissue Kit). Each 96-well plate included two blank wells as negative controls. All samples were subject to genotyping with a set of 18 microsatellites that are used in the molecular genetics laboratory at the Institute of Marine Research for Atlantic salmon genetics projects (e.g., Glover *et al.* 2010, 2011, 2012). These loci were amplified in three multiplexes, using standard protocols for fresh tissues (full genotyping conditions available from authors upon request); *SSsp3016* (Genbank no. AY372820), *SSsp2210*, *SSspG7*, *SSsp2201*, *SSsp1605*, *SSsp2216* (Paterson *et al.* 2004), *Ssa197*, *Ssa171*, *Ssa202* (O´Reilly *et al.* 1996), *SsaD157*, *SsaD486*, *SsaD144* (King *et al.* 2005), *Ssa289*, *Ssa14* (McConnell *et al.* 1995), *SsaF43* (Sanchez *et al.* 1996), *SsaOsl85* (Slettan *et al.* 1995), *MHC I* (Grimholt *et al.* 2002) and *MHC II* (Stet *et al.* 2002). PCR products were analyzed on an ABI 3730 Genetic Analyzer and sized by a 500LIZ™ size-standard. The raw data was controlled manually twice before export for statistical analysis (see Fig. 1). The chromosomal position of these markers and thus their power to discriminate mitotic and meiotic gynogenetics is being investigated.

After tagging and sampling the experimental fish (including 45 control fish) were transferred to three tanks for further ongrowing. In August 2013, 267 confirmed double haploid fish (mean weight 626 grams) are being reared on and are potential clone founders.

The phenotype of these fish are being followed with emphasis on deformities (individuals with skeletal deformities like short opercula (SO), short trunk (STR), short tail (STA), lower jaw (LJ) or upper jaw (UJ), but individuals with these deformities are surprisingly few (18 individuals). Also we do not know the cause of these deformities and whether they can be brought on into the clonal lines.

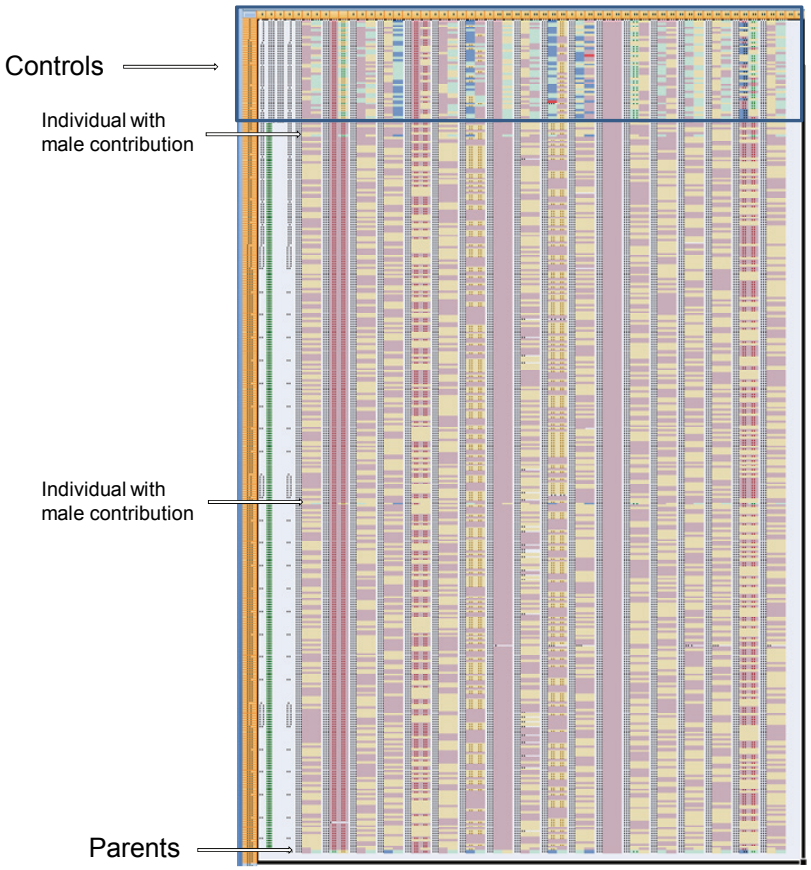


Figure 1. Microsatellite analysis of 2 parents and 362 offspring. The first 45 individuals (controls) were fertilized with diluted, but unirradiated milt. The others were fertilized with UV-irradiated milt and pressure shocked. Sire (sperm) alleles are presented in blue and green. Only two individuals from the putative mitotic gynogenetic groups (arrowed) showed any paternal contribution.

More surprisingly we found that some of the gynogenetic double haploid individuals have a phenotype with very few spots (between 0 and 40 spots) and as many as 72 of the remaining individuals have no spots (Fig 2). Whether the spottedness is related to any other trait and/or whether this observation is coincidental (the eggs come from one mother), will be closely investigated on the 2012 population from where we have progeny from 8 females and more than one thousand potential double haploid clone founders.

Other more obvious phenotypic traits that are being followed are length and weight and condition factor. Condition factor as a possible indicator of adiposity can be an interesting and important phenotypic trait. Figure 3 shows examples of four individuals measured on 28th of November 2012 and 17th of June 2013. Individuals that have a high condition factor in Nov 2012 also have a high condition factor in June 2013. If this 'body shape'/adiposity factor can be brought on into the clonal lines it will be a very interesting phenotypic trait.

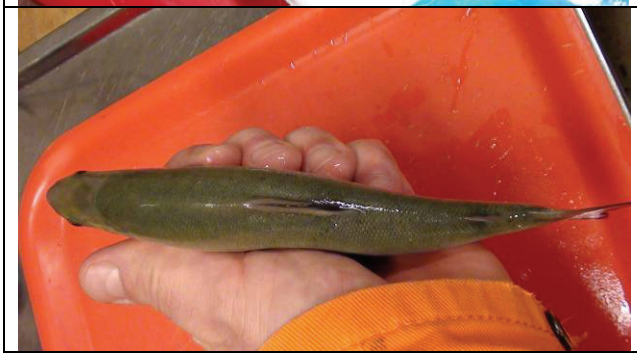
	'Control' salmon from 2011 experiment. The salmon is progeny from a cross between a normal female and untreated sperm from a normal male. The eggs were incubated without pressure treatment. The control fish all had a normal spotted phenotype
	Unspotted gynogenetic double haploid phenotype

Figure 2. Examples of biparental control (upper, normal spotted phenotype) and G1 (lower, unspotted) Atlantic salmon.

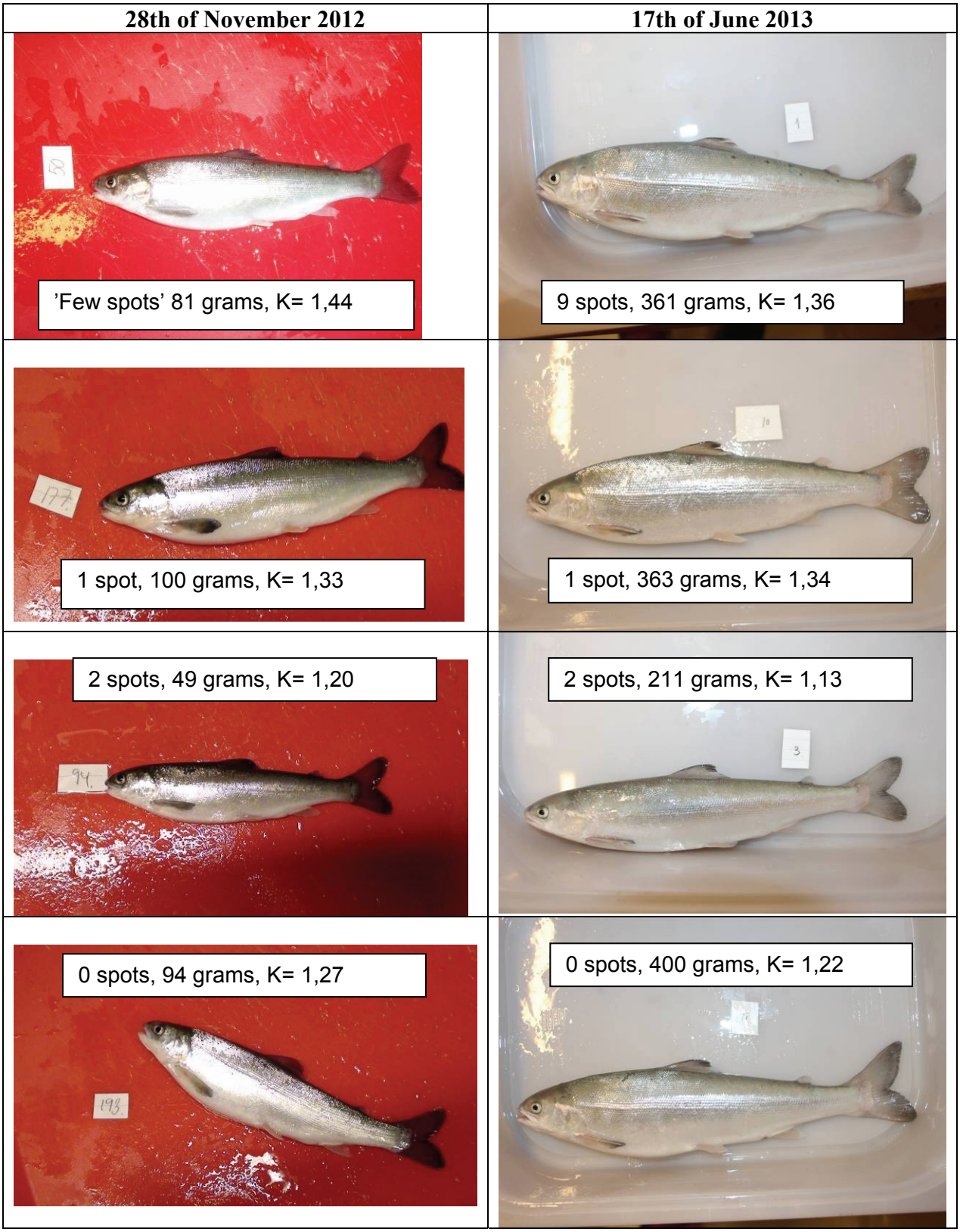


Figure 3. Repeated assessment of the same G1 Atlantic salmon individuals for spottedness and condition factor (K).

2. European sea bass

Objectives

Produce isogenic lines for the sea bass, *Dicentrarchus labrax*

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. The lines will be characterized as follows:

- The uni-parental origin of the first generation and the isogenic status of the second generation will be verified by microsatellite markers (INRA).
- Fish performances will be evaluated by the following variables: growth (weight, length), quality (intra-muscular fat content using a TorryFatmeter®, morphology), sex ratio and reproduction capacities (age at puberty, fertility, sperm quality).

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 2012(M12-M14): optimization of sea bass eggs UV treatment to produce the first mitotic androgenetic offspring.
- April-May 2012(M14-M15): production of the first mitotic gynogenetic G1 offspring (called G1-2012).
- Since May 2012 (M15-M30): rearing of the different G1-2012 offspring.
- December 2012 to April 2013 (M22-M26): optimization of sea bass eggs UV treatment to produce androgenetic offspring.
  111111
- March 2013 (M25): production of the second mitotic gynogenetic G1 offspring called G1-2013.
- Since March 2013 (M25-M30): rearing of G1-2013 offspring in "common garden" tanks.

2.1 Androgenesis

Material 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.
- 6 UV intensities were tested: 7.2, 13.2, 28.8, 42, 60 and 72 mJ/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.

Results

During the 3-4 months of the natural reproduction season that were exploited during these 2 years (2012-2013), 150 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 more than 260 trials were realized (6 doses x 6-7 durations, 3 runs for each combination + specific experiments).

The survival rates of UV-treated groups relatively to controls are reported in Figure 1. Results at 52h and 70h pf were pooled as statistical analyses showed lack of significant differences between these data (p=0.83, ANOVA). Overall, survival fell sharply with increasing UV intensities and durations, in particular at the highest intensities (42, 60 and 72mJ/cm^2/min) where survival dropped to 20% relatively to controls after 1min irradiation only. At the lowest UV intensities (7.2 to 29mJ/cm^2/min) survival rate decreased to less than 10% when eggs were irradiated up to 6 min, before reaching 0% between 10 and 12min. The flow-cytometry analysis revealed that most UV-treatments were ineffective at inactivating the maternal genome and yielded only diploid larvae whereas a small percentage (14%) of haploid larvae (3 on 21 hatched larvae) was observed only at 60mJ/cm²/min with 1 minute irradiation.

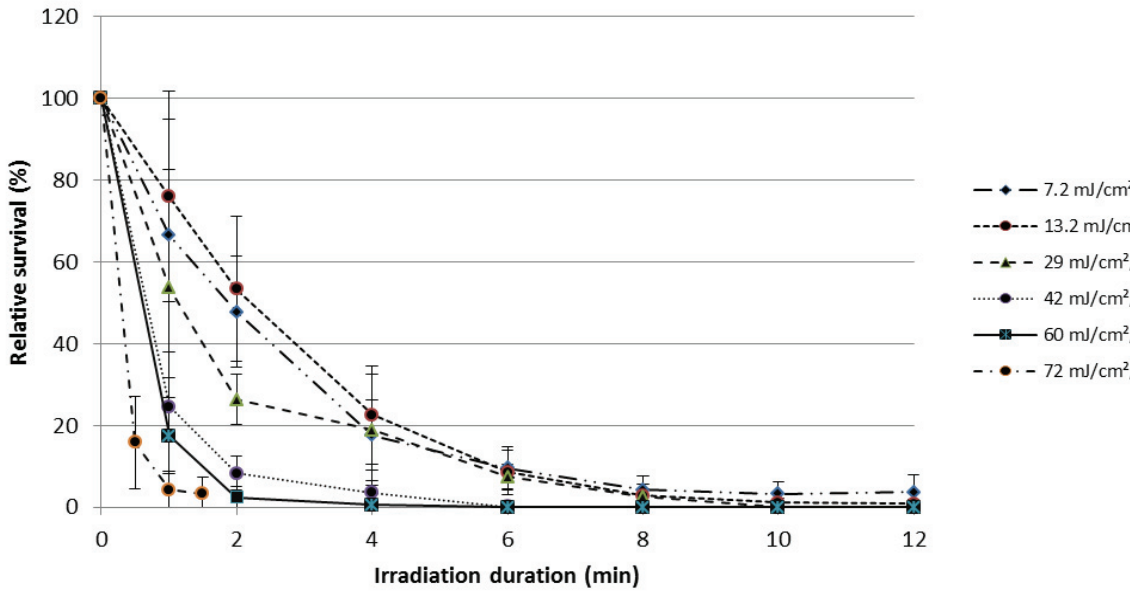


Figure 4. Percent survival of groups exposed to different UV rays in the range 7.2 – 72 mJ/cm^2 for 0-12 min. Relative survival rates based on control fertilization rate and an average of the survival rates (embryonic development) estimated 50h and 74h post-fertilization (n=2400, n=200 per treatment). Fertilization rates in controls ranged 40-70%. Error bars represent standard deviations of means.

The combination of UV intensities and durations tested in the present work covered a large range of irradiation doses shown to be effective at inducing androgenesis in other species (see review by Komen and Thorgaard, 2007). In sea bass, the yield of putative UV-induced androgenetic haploids was unexpectedly low. Several hypotheses can be put forward to explain the results. These include the presence of UV screening compounds in the eggs providing protection at least against low and medium radiation intensities followed by active DNA repair processes, or the expression of recessive paternal mutations at the haploid state affecting survival of androgenetic embryos. Further work on the chemical and absorbance characteristics of egg extracts and molecular characterization of embryos from UV-treated eggs may provide some answers.

Following steps

- UV screening compounds characterization using microspectrophotometry and HPLC (In Tromso from October 9 to November 8 2013).
- UV endonuclease on eggs and larvae to characterize DNA damages.
- UV induction of androgenetic haploids on Tilapia eggs to validate experimental devices.

2.2 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.

Material and methods

The following protocol was used to produce mitotic gynogenetic offspring:

- Sperm stripped 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 mJ/cm²) according to Peruzzi and Chatain (2000).
- Females were stripped and treated separately, only the good quality spawns with a consequent volume of eggs being used. The fish populations originated from domesticated Atlantic lines, domesticated Mediterranean lines 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 offspring.

Results

G1-2012

On the 25 batches of mitotic gynogenetic offspring realized in April-May 2012, 6 are currently on-growing. They are presently aged 15-16 months according to the batch, and represent all together 1186 gynogens.

The uniparental origin and homozygous status were analyzed by INRA using 12 microsatellites markers. These markers were usually used for parentage assignment in many

experiments and their validity for determination of homozygosity was first analyzed on meiotic gynogenetic progenies and their recombination rate determined. On the 12 markers used, 11 are relevant for homozygous status determination.

Table I. Recombination rates estimated for each microsatellite marker. Means are ranging between 0 (no recombination) and 1 (maximum recombining marker).

	A	B	C	D	E	F
Mean	0,76	0,21	0,02	0,76	0,63	0,74
CI 95%	[0,67-0,85]	[0,04-0,37]	[-0,02 - 0,06]	[0,66 - 0,86]	[0,50 - 0,77]	[0,63 - 0,84]
	G	**H**	**I**	**J**	**K**	**L**
Mean	0,66	0,35	0,97	0,47	0,44	0,78
CI 95%	[0,54 - 0,77]	[0,26 - 0,45]	[0,93 - 1,01]	[0,37 - 0,57]	[0,32 - 0,56]	[0,69 - 0,86]

25 fishes/progeny or all the fishes in the small progenies were analyzed using these 11 microsatellites markers and their homozygous status verified. The results are presented in Table II.

Table 2. Progenies currently on-growing for each dame submitted to gynogenesis. Only the markers showing a maternal heterozygosity are used to characterize the progenies.

	Fishes (nb)	Genotypic status
D1	664	25/25 Heterozygous
D2	36	25/25 Heterozygous
D3	1	Homozygous for 9 relevant markers
D4	1	Heterozygous
D5	2	1 Homozygous for 9 relevant markers
		1 Homozygous for 9 relevant markers but paternal contribution on 1 non relevant marker
D6	482	25/25 Heterozygous, 3 clearly showing paternal contribution

29.7% of the fishes from all progenies show at least 1 malformation (92.3% originated from D1, 0.9% from D2 and 6.8% from D6). The 2 homozygous individuals look completely normal for their phenotypic appearance.

Figure 5. Pictures of the 2 homozygous gynogenetic fishes (G1-2012). The first one is from D3 progeny and the second one is from D5 progeny.

Different kinds of malformations were observed especially on D1 progeny: opercular and/or skeletal malformations and/or also different malformations of the jaws.

Figure 6. Pictures of different malformations observed on G1-2012 progenies (from right to left): opercular malformation, prognathism, absence of lower jaw, hyoid malformation, short body due to spinal fusions and skeletal twists.

Following steps

- Growing of D3 and D5 progenies until maturation.
- Assessment of the ploidy status of the progenies genotyped heterozygous to confirm their diploid status and decide whether they should be killed.

G1-2013

10 batches of putative mitotic gynogenetic offspring are currently on-growing in “common garden” tanks. Around 3200 fishes: 1600 controls and 1600 putative gynogenetic fishes are growing and weighted around 3g at the beginning of July. Controls and gynogenetic individuals are growing in triplicates since the beginning of larval rearing (one day before hatching). The aim of this experiment is to compare growth and malformations between the 2 treatments and to evaluate sex-ratio. These putative mitotic gynogenetic offspring are also the second batch of G1.

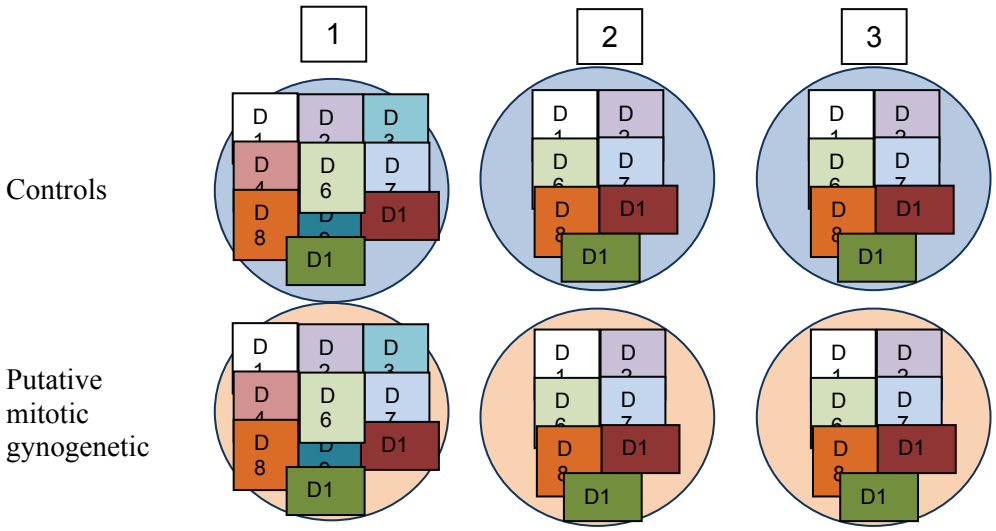


Figure 7. *Schematic representation of the rearing tanks of controls and putative mitotic gynogenetic progenies from the different dames. Each dame was crossed with a sire (sperm UV irradiated for gynogenesis and untreated for controls). 3 progenies are only reared in tanks 1 because the gynogenetic survival eggs were insufficient to provide all tanks number.*

The first biometries done at 4, 12 and 33 days after hatching showed no significant differences in growth pattern and malformation ratios between gynogenetic larvae and controls. The only significant result is the expected high mortality of gynogenetic treated eggs and hatching larvae: 96h post fertilization, the gynogenetic eggs survival rate is about half that of controls. Hatching is also a critical phase and the larval rearing tanks of controls had to be equalized twice during the first 3 months of the experiment.

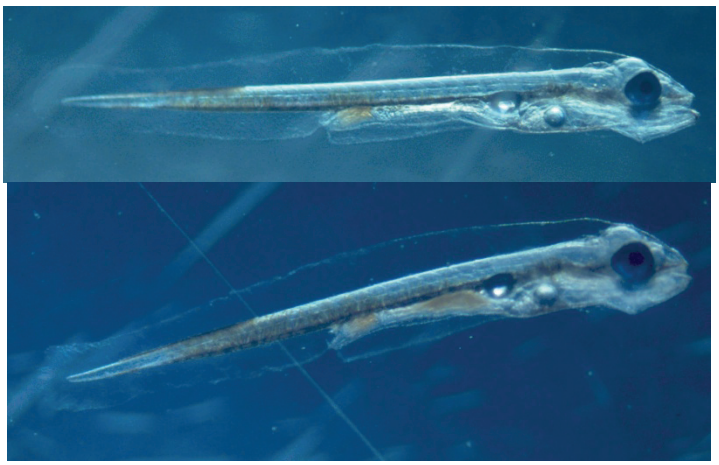


Figure 8. A control diploid larva and a putative mitotic gynogenetic larva at 12 days after hatching. Both show an inflated swimbladder, a well developed eye and a mouth opening.

Following steps

- Growing in “common garden”: 3 tanks of the putative mitotic gynogenetic progenies and 3 tanks of controls until maturation to analyze sex ratio
- Genotyping to assess the homozygous status of the gynogenetic individuals and to assign parentage to all progenies
- Biometries to characterize growth pattern differences and malformations

3. Common carp

Some A1 common carp were produced at HAKI, but died (along with G1 common carp).

3.1 Verification of andro/gynogenetic status

Surviving putative A1 and G1 common carp (n=181) originating from VURH were genotyped for 14 microsatellite markers, as well as 26 potential parents.

The following crosses where performed in VURH:

M72 ANDROGENESIS

MALE		UV irradiated eggs of	
P.I.T.	sample	P.I.T.	sample
2019	2019	1365	7
5662	? - 22, 23, 24, 25, 26,	3257	10
4381	27, 28, 31, 32, 34, 37, 39, 40	3384	3384

ROPSHA ANDROGENESIS

MALE		UV irradiated eggs of	
P.I.T.	sample	P.I.T.	sample
8118	36	5829	8
5291	30	3291	12
4574	45	4207	9

M72 GYNOGENESIS

FEMALE		UV irradiated sperm of	
P.I.T.	sample	P.I.T.	sample
4207	9	4574	45
7595	1	4574	45
5829	8	4574	45

ROPSHA GYNOGENESIS

FEMALE		UV irradiated sperm of	
P.I.T.	sample	P.I.T.	sample
7552	20	8118	36
9214	19	8118	36
1365	7	5291	30

For M72 androgenesis, the traceability of the males used for crosses with females 5662 and 4381 was lost, but the sires potentially used were re-collected later on (males 22 to 40 in the table)

181 putative andro and gynogenetics were analyzed at INRA for 14 microsatellites, 8 of which were evaluated before for their distance to centromere (hence their ability to distinguish between meiotic and mitotic gynogenesis)

Among the 181 fish analyzed, the results were the following:

- 99 androgenetics (A1):
  - 43 from Ropsha male 8118
  - 56 from M72, among which all were compatible with male 2019, and 5 were also compatible with male 44. As no individual was compatible with male 44 only, the most parsimonious conclusion is that all 57 androgenetics are from male 2019
- 43 mitotic gynogenetics (G1):
  - 39 from Ropsha female 7552
  - 1 from Ropsha female 9214
  - 3 from M72 female 5829

Among the unexpected genotypes, 23 individuals were found biparental for female Ropsha 9214 and male 8118, the sperm of which was irradiated for Ropsha gynogenesis with female 9214. This shows that the irradiation was not complete. Similarly, 5 biparental individuals were found for Ropsha female 7552, also with male 8118 which was the male used for this gynogenesis, with the same conclusion on sperm irradiation. One bi^parental individual with Ropsha female 1365 and male 5291 led to the same conclusion

One meiotic gynogenetic (showing heterozygosity at 5 loci where the dam was heterozygous) was found for Ropsha female 9214, a phenomenon commonly observed to some extent in mitotic gynogenetic trials. This one cannot be kept as a founder.

Two individuals showed an uncertain genotype, one which was compatible with M72 androgenesis for male 2019, but with one heterozygous locus, and one which was compatible with M72 gynogenesis for female 4207, but also with one heterozygous locus.

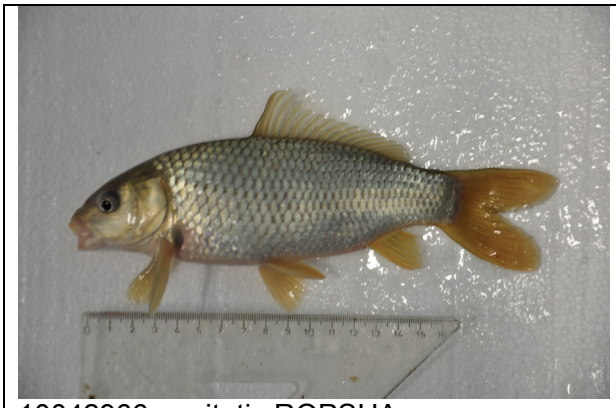
Three fish were homozygous at all loci, compatible with Ropsha androgenesis with male 8118, except for one locus (MFW16) where the genotype was homozygous but not compatible. Their suitability as potential founders can be questioned.

Finally, one fish was compatible with Ropsha male 8118, but not any female. All its alleles were from male 8118, but it was heterozygous at 6 loci out of 9 where this male is heterozygous. If it were an unreduced (diploid) spermatozoon, the probablility of such a genotype would be low (1.5%). One could also think of a dispermic individual – or more likely, of a contaminated fin sample containing DNA from two androgenetic individuals.

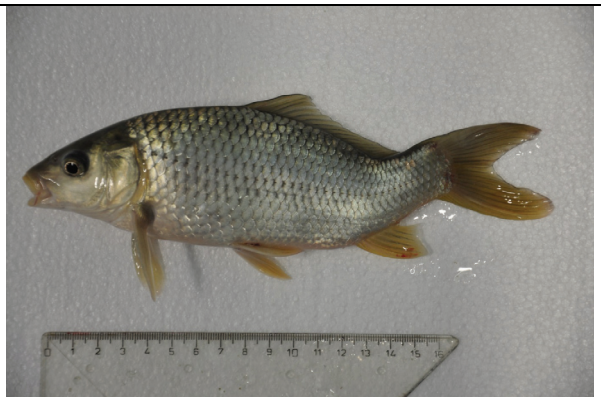
3.2 Phenotyping of the potential founders

The genotyped individuals still alive in February 2014 were also phenotyped for phenotypes that are measurable on live fish, i.e. body weight, head length, body length, total length, height and width. They were also individually photographed. Phenotyping for muscle fat content with a Distell fat-meter should also be done when the fish reach a sufficient size.

Below are some pictures of several A1 and G1 fish, showing their general appearance.



10042966 – mitotic ROPSHA



10042760 – mitotic ROPSHA

5348510 – androgenetic M72

10043922 – androgenetic M72

10044088 – androgenetic ROPSHA

10151569 – androgenetic ROPSHA

Many fish are rather deformed, but some biparental fish also are, so it seems to be a consequence of larval rearing in aquaria rather than of gynogenesis or androgenesis.

Concerning biometric measurements, the results are summarized in the table below (mean and coefficient of variation (%) of the trait):

trait	**M72 androgenetic**	**ROP androgenetic**	**ROP mitotic gynogenetic**	**ROP biparental**
Body Weight	132 g (49%)	137g (131%)	131g (29%)	269g (57%)
Head Length/Total length	0.192 (6.9%)	0.210 (7.3%)	0.220 (8.6%)	0.201 (9.3%)
K= BW/L^3	1.97 (11.6%)	1.86 (11.4%)	1.85 (12.2%)	1.86 (6%)
Height/Total length	0.26 (10.1%)	0.27 (10.1%)	0.27 (6.1%)	0.27 (5.2%)
Width/Total length	0.16 (6.2%)	0.17 (5.8%)	0.16 (5.6%)	0.17 (5.1%)

Two features are clearly apparent: body weight is approximately half in gyno or androigenetic fish compared to biparental fish. What is also clear is that the CV of body weight is high (approximately twice the expected value for this species) in all genotypes, probably a consequence of the rearing method in aquaria.
The coefficient of variation of condition index (K) is higher in andro or gynogenetic fish, and this is likely to be due to a higher incidence of deformities.
For the rest (head length/body length, an indicator of fillet yield; height or width/total length), all genotypes seem pretty much alike.
However, the high level of variation for all traits makes the potential to select contrasted phenotypes for the next step (foundation of isogenic lines) interesting

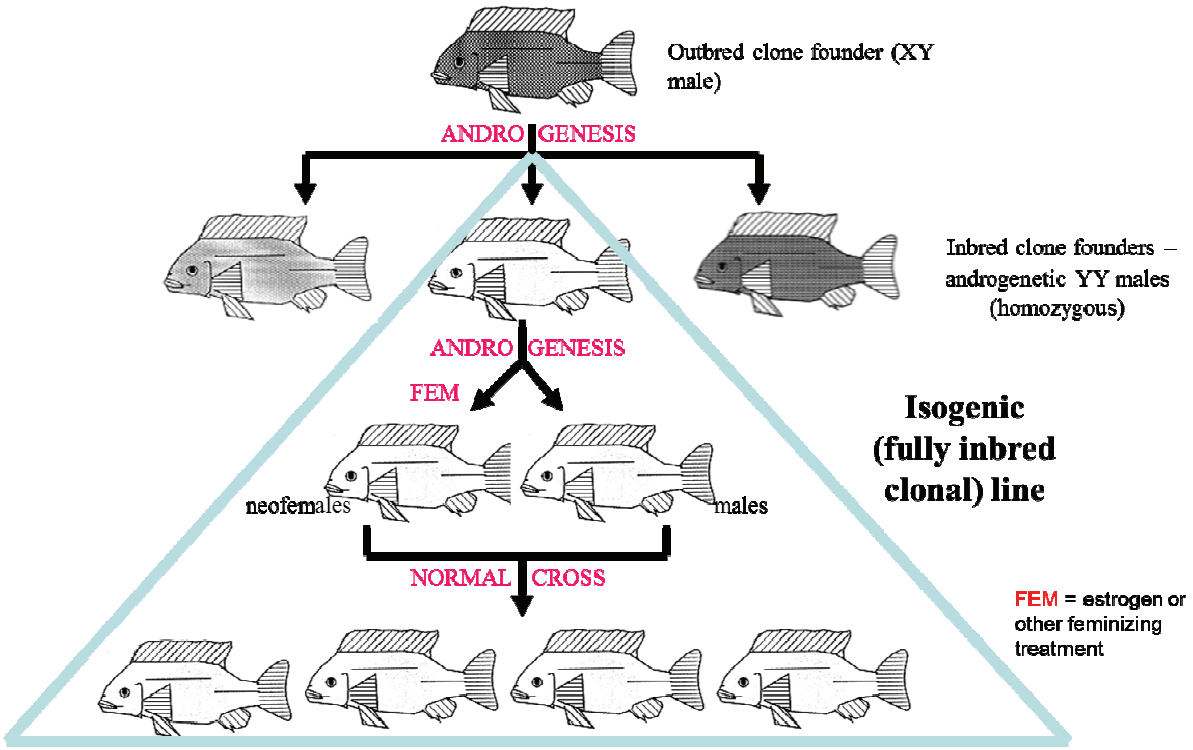
Conclusions

- G1 (mitotic gynogenetic) progeny were successfully produced from outbred founders in all three target species (Atlantic salmon, European sea bass and common carp). These were verified using molecular markers (primarily microsatellites).
- >250 verified G1 Atlantic salmon (2011 breeding season) are available. These show considerable phenotypic variation in spottedness and condition factor.
- Only two verified G1 European sea bass (2012 breeding season) are available. Other surviving fish from the same batch were shown not to be dihaploids. Large numbers of putative G1 European sea bass from the 2013 breeding season are being reared.
- Trials on androgenesis in European sea bass failed due to lack of success in the first step (optimization of UV inactivation of the female genome, leading to production of haploid androgenetic embryos).
- Some A1 common carp were produced at HAKI, but died (along with G1 common carp). Surviving putative A1 and G1 common carp originating from VURH were checked with microsatellite markers, and 99 A1 and 43 G1 founders were identified, and phenotyped.

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)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time		*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW		*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW		
	The contributors (authors) correspond to those indicated in the DOW		
	The Table of Contents has been validated with the Activity Leader		*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL deliverable template (title page, styles etc)		*Available in "Useful Documents" on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable		*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)		*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified		*Ask a colleague with a good level of English to review the language of the text and do a spell-check too.*
	I have sent the final version to the Activity Leader and to the 2nd Reviewer for approval		*Send the final draft to your Activity Leader and the 2nd Reviewer and leave 2 weeks for feedback and final changes before the due date. Once validated by the 2 reviewers, the draft is ready to be sent to the Management Team that will ask for the Coordinator validation and then transfer it to the EC.*