DEVELOPMENT AND APPLICATION OF GENETICALLY UNIFORM STRAINS OF COMMON CARP *(CYPRINUS CARPIO* L.)

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Development and application of genetically uniform strains of common carp *(Cyprinus carpio* L.)

Augustinus B.J. Bongers

Proefschrift

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Abstract

Bongers, A.B.J. 1997. Development and application of genetically uniform strains of common carp *(Cyprinus carpio* L.). Doctoral thesis, Wageningen Agricultural University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.

In this thesis, the development of genetically uniform strains of common carp, *Cyprinus carpio* L. is described. As in research on mammals, the use of genetically uniform fish could increase the quality (replicability, reproducability and repeatability) of experiments. Inbreeding was done by gynogenetic and androgenetic reproduction. With these methods, fully homozygous offspring are produced in only one generation. The principle of these reproduction techniques is to eliminate the genetic contribution of one of the parents by (UV) irradiating the sexual products, followed by artificially inducing diploidy by physical shocks in the developing zygotes. Inbred strains, congenic strains and F1 hybrids were produced. Congenic strains were used to study sex determination in carp. Inbred strains and F1 hybrids were used to study the genetic control of gonad development after a quantitative genetic model had been developed to facilitate estimation of genetic variance. We show that genetically uniform carp strains are suitable as experimental animal model in genetic research and in bioassays (toxicological research). It is obvious that other research areas could also profit from genetically uniform carp strains.

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STELLINGEN

1. Gynogenese en androgenese zijn waardevolle technieken in selectie-programma's van vissen.

   Dit proefschrift

2. In tegenstelling tot hetgeen beweerd wordt door Ihssen et al. (1990) is het bestralen van karper-eieren met UV licht (254 nm) een efficiente methode om de vrouwelijke pronucleus uit te schakelen.

   Ihssen PE, McKay LR, McMillan I and Phillips RB (1990) Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. *Trans. Am. Fish. Soc.* **119**, 698-717.
   Dit proefschrift

3. De toename van de fenotypische variatie, algemeen waargenomen na gynogenetisch of androgenetisch voortplanten, is voornamelijk het directe gevolg van de temperatuurschok.

   Dit proefschrift

4. Gynogenese en androgenese resulteren in ongebruikelijke genetische relaties tussen en binnen groepen nakomelingen. Het zonder meer toepassen van gangbare modellen voor de berekening van genetische variatie is derhalve niet mogelijk.

   Dit proefschrift
   Bijma P, Van Arendonk JAM and Bovenhuis H (1996) Breeding value and variance component estimation for gynogenetic families. *Genetics*, accepted for publication.

5. Het verschil in expressie van het gonadale geslacht is bij lagere vertebraten het gevolg van verschillen in splicing van heterogeen nucleair RNA.

   Baker BS (1989) Sex in flies: the splice of life. *Nature*, **340**:521-524.
   Harry JL, Williams KL and Briscoe DA (1990) Sex determination in loggerhead turtles: differential expression of two hnRNP proteins. *Development*, **109**:305-312.

6. Er bestaan sterke aanwijzingen dat intelligentie-bepalende "major genes" in zoogdieren gelocaliseerd zijn op het X-chromosoom (Turner, 1996). Indien dit ook het geval zou zijn bij lagere vertebraten, komen androgenetisch mannelijke (YY) karpers in aanmerking voor huisvesting onder toezicht.

   Turner G (1996) Intelligence and the X-chromosome. *The Lancet*, **347**:1814-1815.

7. Een carrière aan de universiteit is voor wetenschappers niet aantrekkelijk meer aangezien het klimmen op de wetenschappelijke ladder resulteert in een toename van bureaucratische taken en een afname in wetenschappelijke produktie. De Modernisering Universitaire Bestuursorganisatie (MUB), die voorziet in het aanstellen van een beroepsdecaan dient derhalve door de eerste kamer afgewezen te worden.

8. Naast het gebruik van genetisch uniforme lijnen kan een gedegen statistische scholing van onderzoekers tot een vermindering van het aantal gebruikte proefdieren leiden.

9. "Specialiseren" is steeds meer van steeds minder afweten tot men uiteindelijk van vrijwel niets alles afweet.

Godfried Bomans

10. All animals are equal, but some are more equal than others.

Dit proefschrift

Stellingen behorende bij het proefschrift
"Development and application of genetically uniform strains of common carp, *Cyprinus carpio* L." ,
Augustinus B.J. Bongers,
Wageningen, 14 januari 1997.

Contents

Chapter 1

Introduction to the papers

Scientific research on fish is often hampered by large variation between animals within the same experimental group. In mammalian research, these problems are minimized by using animals from genetically uniform strains. Examples of genetically uniform strains are homozygous inbred strains, F1 hybrids and congenic strains.

Through inbreeding (crossing genetically related animals), homozygous isogenic (genetically identical) inbred strains are obtained. Crossing two homozygous, not related animals yields an (isogenic) F1 hybrid. Congenic strains can be obtained by repeatedly backcrossing offspring, selected for the gene of interest to the same homozygous parent. However, 20 generations of inbreeding are needed before animals can be considered homozygous. In fish, homozygosity can be induced in only one generation by eliminating the genetic contribution of one of the parents and subsequently doubling the (haploid) genome. Artificial gynogenesis (all female inheritance) has been used in a previous research project of the Department of Fish Culture and Fisheries and the Department of Animal Morphology and Cell Biology to generate homozygous gynogenetic inbred strains of common carp, *Cyprinus carpio*. A new research project was initiated at the same departments aiming to produce genetically uniform strains of carp and to investigate their suitability as animal model in reproduction and immunological research. Obviously, the strains generated can also be used in other fields of research, e.g. physiology, endocrinology, and toxicology. This thesis concentrates on uniform carp strains designated for reproduction research.

Before experimental data are presented, an introduction to the use of uniform fish strains is given in chapter 2, since many misunderstandings concerning genetically uniform strains exist. This chapter provides data on the importance of fish in general, and more specific on the importance of carp in experimental animal research. It also reviews methods to produce specific types of uniform strains and it presents their impact on the statistical evaluation of experiments.

In figure 1, the structure of the experimental papers in this thesis is summarized. In chapter 3, the development of androgenetic (all-male) reproduction techniques is described. In theory, androgenetic offsprings contain YY males. Since this genotype is lethal in mammals, their viability had to be demonstrated in homozygous carp (chapter 4).

After gynogenetic or androgenetic reproduction, an increase in the total (phenotypic) variance is observed. Phenotypic variance consists of genetic and environmental (non-genetic) variance. In chapters 5 and 6, the increase in environmental variance was analyzed. Chapter 5 concentrates on environmental factors affecting the variance in androgenetic offsprings. In chapter 6 a detailed analysis on the origin of several types of environmental variance in gynogenetic, androgenetic and (conventional) inbred offspring is presented. In chapter 7,

distribution of genetic variance after gynogenetic or androgenetic inbreeding was analyzed. A quantitative genetic model was developed and shown to be useful when gynogenesis or androgenesis are used additionally in selection programmes. As model parameters, egg quality and age at maturation were used. In chapter 8, uniform strains (F1 hybrids) were produced from parents, selected for age at maturation. In these strains, genetics of testis development was studied.

Finally the production, application and genetic monitoring of genetically uniform carp strains is discussed in chapter 9.

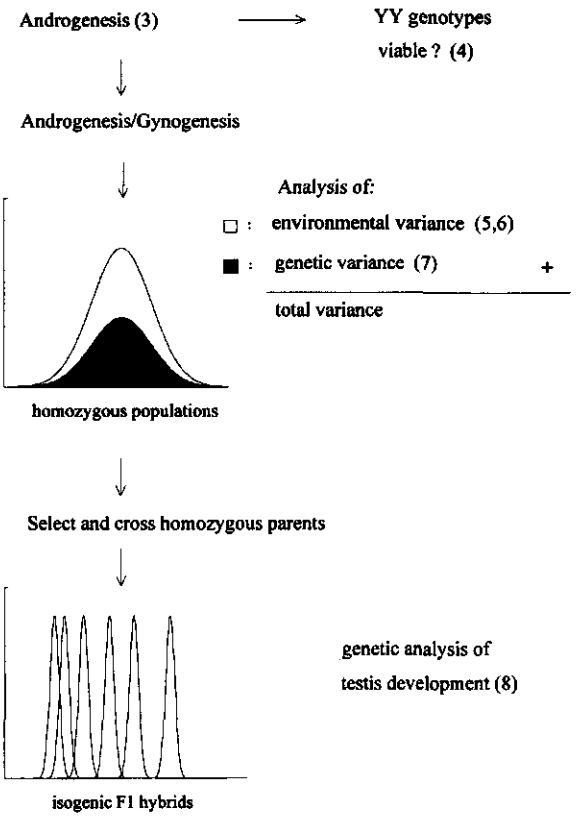

Figure 1: Structure of this thesis. Figures between brackets refer to chapters.

Chapter 2

Clones of common carp in experimental animal research

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submitted for publication in: **Laboratory Animals**

Summary
Fishes are widely used in numerous fields of basic and applied research. Currently, they are the third laboratory animal group in numbers, and will become increasingly important. Common carp is a major species in both aquaculture and research. Inbred strains of carp by gynogenetic (only female inheritance) and androgenetic (only male inheritance) reproduction techniques were developed at our university. With these methods, homozygous animals are produced in one generation and we present the production of homozygous inbred and F1 hybrid strains of common carp.
As in mammalian research, using genetically well defined fish is a methodological necessity since in outbred strains (1) repeatability between experiments is low, (2) high levels of inbreeding may have accumulated and (3) high intrastrain variability might obscure treatment effects. Within inbred strains, the variation is reduced and as a result, less animals (compared to outbreds) are necessary to obtain statistically significant results. We illustrate this with experimental data from a F1 hybrid and partly outbred strain of common carp, both subjected to an antibiotic treatment resulting in reduced gonadal growth. Results obtained from a single inbred strain should be generalized with the use of a panel of inbred strains. We show that optimal allocation of animals between and within inbred strains depends on the ratio (variation between strains) : (variation within strains). When selecting a panel of inbred strains, attention has to be paid to genetic relations between strains to avoid testing within a limited genetic range. It should be considered that in inbred strains, (genic) dominance and interaction effects are absent, due to the absence of heterozygous genotypes,.
In general, variation within inbred strains will be reduced for traits with high degree of genetic determination. However, in inbred strains of carp produced by gynogenesis or androgenesis, the chromosome manipulation treatment induces considerable (environmental) variation. By using F1 hybrids or carp, derived from crossing homozygous clonal siblings this source of variation can be avoided. Still, variation in F1 hybrids of carp is relatively large and varies greatly between strains when compared to inbred strains of laboratory rodents. It is assumed that their poikilothermic nature makes them more susceptible to environmental variation. Using inbred fish lines will increase experimental quality and leads to a more efficient use of experimental animals.

Fish are widely used in the field of neurobiology, endocrinology, immunology, developmental biology, aquatic toxicology and cancer research. The advantages of fish as experimental animal are numerous. Great diversity between species exists, they are highly fecund and, in most cases, they have large eggs with external fertilization (Powers, 1989). At present, fish are the third laboratory animal group in numbers (figure 1). They are used mainly in routine toxicity testing (60 %) of which the majority is required by law. In contrast, in mammals routine toxicity tests only account for 20 % of the number of animals used. For two main reasons, fish will become increasingly important as laboratory animals. First, there is a gradual shift in using lower vertebrates, since legislation in the European Community is directing towards the use of animals with low degree of neurosensitivity (EC council directive 86/609/EEC, 1988). Secondly, since regulations regarding environmental safety become stricter, more safety tests are required for newly developed, as well as existing chemicals. In the European Community, materials whose manufacture, transportation, use or disposal will involve a potential of reaching surface water will normally require at least minimal testing of their effect on aquatic organisms (EC council directive 79/831/EEC, 1981).

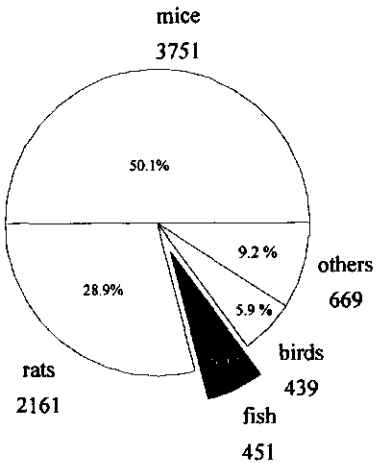

Figure 1: Total numbers (in thousands) of laboratory animals used in Germany (1993), Great Britain (1991), Switzerland (1991) and the Netherlands (1991) according to laboratory animal statistics. Fish is the third major group after mice and rats.

While in mammalian research, inbred strains of in particular mice and rats made substantial contributions to many areas of biomedical research (Festing, 1979), very little demands are made thusfar on fish. The only requirements concern in most cases health status and size homogeneity. Frequently, experimental fish with unknown genetic background and raising history are used. Some genetically uniform strains of fish have been produced through conventional inbreeding and have shown to be suitable for immunological, radiation and genetic research (medaka, *Oryzias latipes*: Hyodo-Taguchi and Egami, 1985; platyfish, *Xiphophorus maculatus*: Kallman, 1984). In conventional inbreeding, approx. 20 generations of full sib mating are needed to obtain (near) homozygous animals. However, because most fish have external fertilization, manipulation with the sexual products is possible and fully homozygous (inbred) fish can be produced in only one generation. This enables the rapid production of inbred strains, with increased possibility to directly select specific genotypes.

Cypriniforms are the most diverse group of freshwater fishes, consisting of approx. 3000 species. They are cultured on all continents, except South America (Billard and Marcel, 1986). In 1992, a total of 6.6 million tons of carp species was produced worldwide, representing a value of 8.1 billion US $. Carp species account for 70 % of the world finfish production (FAO, 1995).

Besides having a prominent position in aquaculture, cyprinids are also an important laboratory animal model (fig. 2). In fundamental research and bioassays (mainly toxicity tests), Cypriniforms are used in at least 25 % of all studies. Common carp (*Cyprinus carpio*) is the most frequently used among the Cypriniforms. For these reasons, the development of inbred strains of common carp was initiated at our university. In this paper, we review methods to produce inbred strains of common carp and their use as experimental animal model.

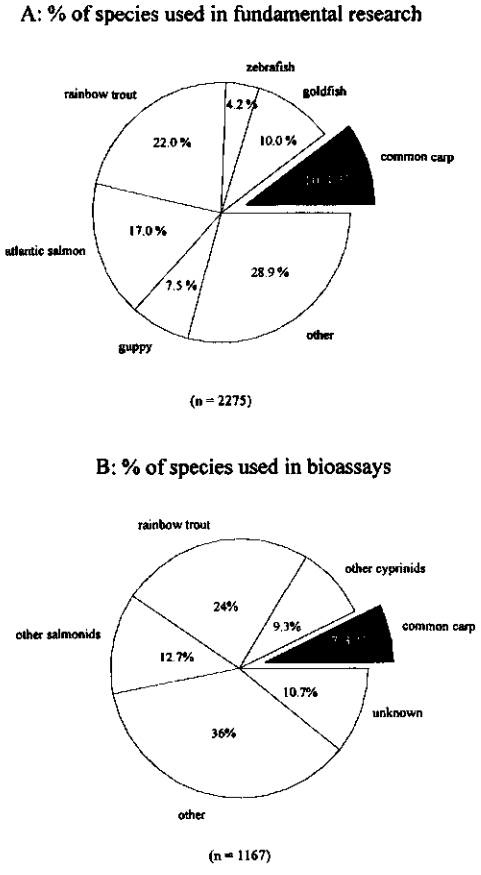

Figure 2. Important fish species as laboratory models, separately presented for fundamental research areas (A) and in bioassays (B). In both areas, common carp is the most important model among the cyprinids (10.4 resp. 7.3 %). Data were obtained by electronic literature search (MEDLINE, 1966-1993; TOXLINE: 1981-1993; BIOSIS: 1990-1993; BEAST-CD: 1972-1993; VET-CD: 1972-1993). n = number of publications.

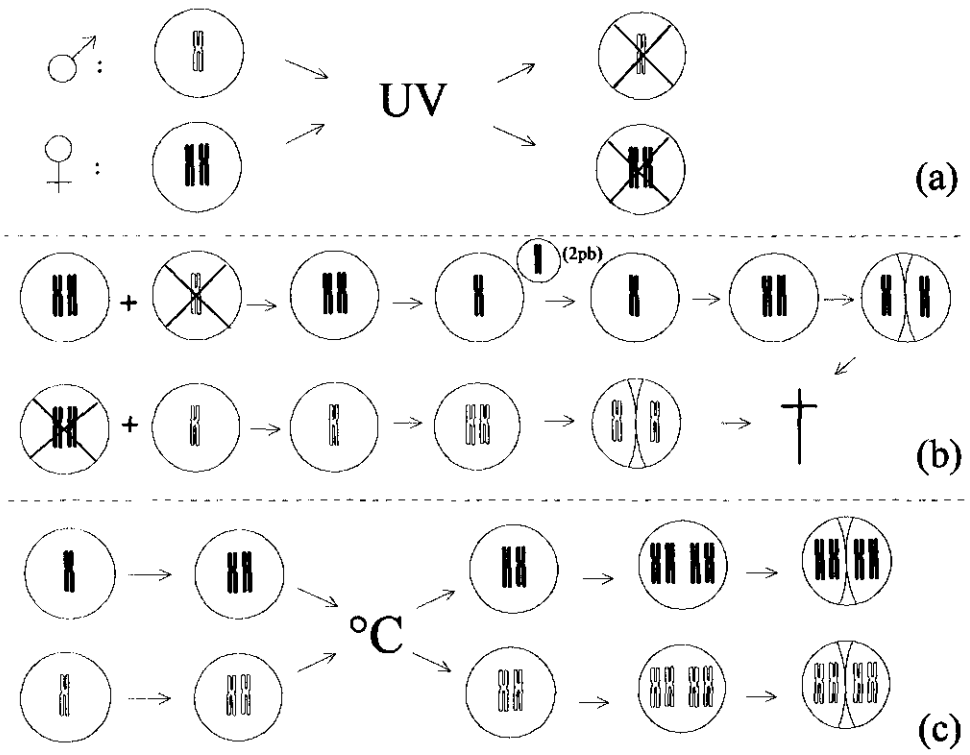

Figure 3. Principle of chromosome manipulation techniques to produce homozygous individuals of common carp. (2pb) = second polar body, produced at meiosis II. Gynogenesis by retention of the second polar body ("heterozygous gynogenesis") is possible through similar techniques (not shown).
† = non-viable haploids. °C = heat shock to induce diploidy.

(a): UV-irradiating sperm or eggs results in genetically inactive DNA.

(b): Fertilizing intact eggs with irradiated sperm (gynogenesis), or irradiated eggs with intact sperm (androgenesis) yields haploid zygotes that start development, but die around hatching.

(c): After the initiation of haploid development, DNA is replicated. To prevent nuclear division, a heat shock is applied at the metaphase of this first mitotic division. This results in the initiation of a new cell cycle, starting (again) with DNA-replication. The zygote is now diploid and fully homozygous.

Production of inbred carp strains

Inbreeding is the mating of related individuals. The coefficient of inbreeding is defined as the chance that the two genes at any locus in an individual are identical by descent. Using conventional reproduction, inbred strains can be produced by repeated full sib mating. After 20 generations, strains can be designated as being inbred (F = 0.986), as decided in 1952 by the Committee on Standardized Genetic Nomenclature for mice. However, residual genetic variation will remain present.

Induction of homozygosity

In fish, complete homozygosity can be attained in only one generation, thus saving a significant amount of time and money. Figure 3 summarizes the production of homozygous common carp. The principle is to eliminate the genetic contribution of one of the parents, followed by artificially doubling the haploid genome. This can be achieved by treating gametes before fertilization with ionizing radiation like gamma-rays or ultraviolet irradiation (fig 3a). Ionizing radiation causes breakdown of chromosomes into small fragments. UV-irradiation initiates the formation of thymidine-dimers in adjacent base-pairs, rendering the DNA inactive (Thorgaard, 1983; Friedberg, 1985). After fusion of gametes of which one parental genome is inactivated, haploid zygotes are produced (fig.3b). Without any further treatment development proceeds, but haploids die around the moment of hatching. However, the haploid state of the zygote can be changed into a diploid state by suppressing the first cleavage using physical shocks (temperature, pressure or a combination of the two), applied at the metaphase of the first mitosis (fig. 3c). After this treatment, a new cell cycle is initiated, starting with DNA-replication. Because an exact copy of the DNA is made, all homologues are fully identical, thus a 100 % homozygous individual will be generated. Gynogenesis (all female inheritance) involves the irradiation of the paternal genome. This technique has been extensively studied and homozygosity can now be induced in several species (for review, see Ihssen et al., 1990). Androgenesis (all male inheritance) is achieved after irradiating the maternal genome. Thusfar, it has been applied with much less success than gynogenesis. Irradiating eggs is more complicated than irradiating a sperm suspension, due to the relatively large size and adhesive chorion. However, we developed a simple and safe method of irradiating common carp eggs with UV (Bongers et al., 1994). Androgenesis is valuable since phenotypic effects of (maternal) cytoplasmic constituents can be studied (Thorgaard, 1986; Bongers et al., 1995), genotypes can be recovered from cryopreserved sperm (Scheerer et al., 1991) and the generation interval can be decreased since in general, male fish sexually mature earlier than their female conspecifics (Horváth and Orbán, 1995).

Genetic markers

To proof the absence of genetic contribution of one of the parents, dominant morphological traits are most frequently used as "genetic markers". For example, in gynogenesis in common carp, irradiated sperm of scaled carps (dominant trait) is used to fertilize eggs from scaleless females (recessive trait). Absence of scaled individuals among gynogenetic offspring confirms elimination of the male genome (Komen et al., 1991). In androgenesis, sperm of males with a recessive mutation causing absence of normal melanophore development ("blond") is used, whereas eggs from females without this mutation are used. As a result, only androgenetic offspring shows the blond phenotype (Bongers et al., 1994). Proof of homozygosity can be achieved by Mendelian segregation of alleles, heterozygous in the parent (Komen et al., 1991), isozymes (f.e. Scheerer et al., 1991), skin transplantations (Komen et al., 1990) and recently also by microsatellite markers (Crooijmans et al., submitted).

Production and maintenance of inbred strains

Inbred strains of fish (i.e., fully homozygous clones) can be produced by gynogenetic or androgenetic reproduction of homozygous individuals (fig. 4). However for diploidy induction (application of physical shocks), high quality eggs are needed (Komen et al., 1992a; Quillet, 1994; Bongers et al., 1996a). Fertility in inbred animals is in general lower than in outbred animals due to inbreeding depression (Falconer and Mackay, 1996) and manipulation with eggs from homozygous females to produce inbred strains or to maintain important genotypes frequently fails. However, we found that egg quality was genetically determined to a large extent in homozygous common carp families. This indicates that selection of homozygous females with high egg quality is possible (Bongers et al., 1996a). Established inbred strains can be maintained by sex-reversing part of the progeny: in many fish species, the phenotypic sex of individuals can be altered since the differentiation of the gonads into a testis or ovary occurs after hatching (for review, see Pandian and Sheela, 1995). For example, genetic females of common carp can be converted into phenotypic males after feeding a diet containing male sex steroids during the process of sex differentiation (Komen et al., 1989). Thus, crossing two identical homozygous genotypes, of which one is hormonally sexreversed, will yield a new stock of the initial genotype.

F1 hybrids and congenic strains

F1 hybrids (heterozygous clones) are produced by crossing two homozygous (not related) individuals (fig. 4). From F1 hybrids, recombinant inbred strains can be produced by gynogenesis or androgenesis, and repeated backcrossing of a F1 hybrid to an inbred strain yields a congenic strain (not shown in fig. 4).

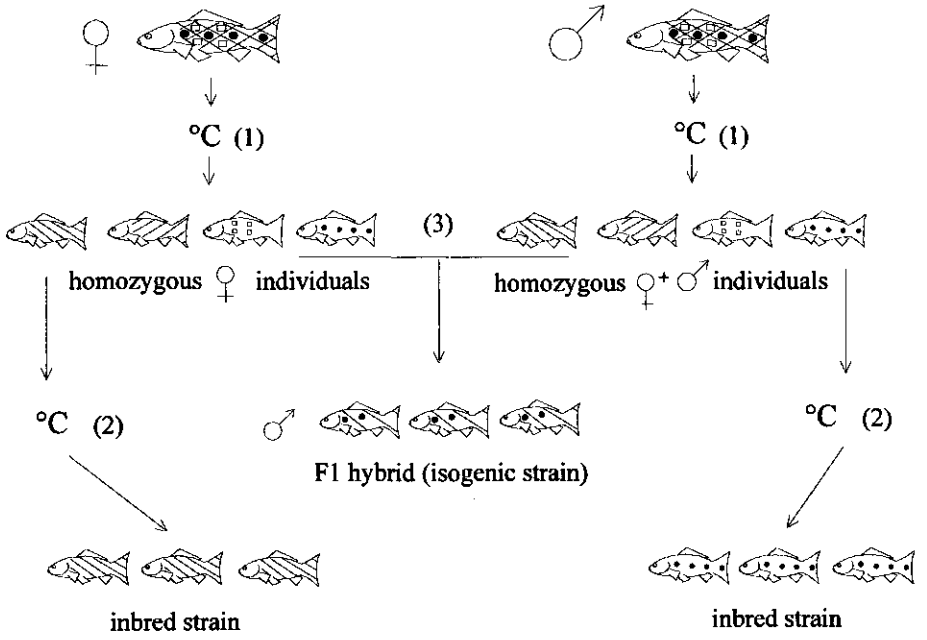

Figure 4: Schematic presentation of the production of inbred strains and F1 hybrids in carp in 2 generations. °C = temperature shock to artificially induce diploidy.

(1): Females (XX) and males (XY) are gynogenetically resp. androgenetically propagated yielding homozygous individuals. Gynogenesis yields only females (XX), androgenesis yields 50 % females (XX) and 50 % males (YY).

(2): Inbred strains can be produced by gynogenetically or androgenetically propagating homozygous females resp. males.

(3): Crossing a gynogenetic (XX) female with an androgenetic (YY) male yields all-male isogenic F1 hybrids (XY).

Congenic strains offer the possibility to examine single (major) gene effects on a standardized genetic background (Festing, 1979). We used congenic strains to study sex determination in common carp. In this species, sex determination is of the XX/XY (male dominant) system (Komen et al., 1992b; Horváth and Orbán, 1995). We repeatedly crossed male (XY) offspring to the same homozygous gynogenetic (XX) inbred strain. Stable 50 : 50 sex ratios were obtained in all backcrosses, indicating the presence of dominant maleness-inducing genes (Bongers et al., 1996b). Males from the sixth backcross were used to produce homozygous androgenetic YY males. These YY males are now used in the production of all-male (XY) F1 hybrids by crossing them with XX-females. All-female F1 hybrids are produced after crossing a XX-female with a XX-(hormonally sexreversed)-male.

Use of outbred vs inbred strains

Outbred strains are defined as a closed colony (> 4 generations) of animals with a limited increase of the coefficient of inbreeding (< 1 % per generation, van Zutphen, 1993) and are assumed to be genetically variable within the colony. There are two main objections to the use of outbred strains. First, especially in small populations maintained as closed colonies for long periods, high levels of inbreeding may accumulate in outbred stocks even when the mating of close relatives is avoided (Festing, 1993). When the level of inbreeding is kept within acceptable limits, genetic drift (reduction in gene frequencies, due to for example unintentional selection) can still result in reduced genetic variation. Secondly, if genetic variability in outbred strains is high, the increased experimental "noise" could obscure true treatment effects or is mistaken for a treatment effect. High quality animal experiments have 1) a high replicability (= low variation between replicates during a single measurement), 2) a high repeatability (= low variation between tests within the same laboratory) and 3) a high reproducability (= low variation between tests from different laboratories) (Dave, 1993). It is obvious that using outbred strains will especially decrease reproducability. Therefore, using genetically well defined animals is a methodological necessity (Festing, 1992).

Statistical implications of using inbred strains

Inbred strains do not represent genetic variation. Since genetic differences in response to chemicals are probably ubiquitous (Lovell, 1993), results obtained from toxicological screening one single inbred strain should be generalized with the use of a panel of (not related) inbred strains (Festing, 1975; 1979; Haseman and Hoel, 1979; van Zutphen, 1993). For the analysis of quantitative traits, Festing (1975, 1992) and Lovell (1993) promote the use of factorial designs, where two or more experimental variables (or factors) are studied at the same time. Factors can

be for example treatment (dose level), sex and strain. The main advantages of a factorial design include the study of interaction effects (e.g., sex × treatment; strain × treatment) and optimization of resources. Differences between strains can provide information on the physiological mechanisms of action in toxicological effects (Lovell, 1993). Although inbred strains are often charged to be unnatural, physiological processes like growth, metabolism etc. are intrinsically the same as in outbred strains (Festing, 1979).

Increase in statistical power

In testing statistical hypotheses, two types of error in decision-making may occur. A type I error stands for rejection of the null hypothesis (H_0) when it is true. A type II error stands for non-rejection of the null hypothesis when it is false. The probability of a type I error is denoted by α. The probability of a type II error is denoted by ß. The power of a test, i.e. the rejection of the null hypothesis when it is indeed false, then equals 1 - ß.

A reduction in the variance between animals within the same experimental group increases statistical precision, and as a consequence, decreases the chance to commit a type I or II error. Assume the following example: a number of animals (n) are being sampled on which a normally distributed trait y is measured. A test is performed to determine whether the sample with mean = $\hat{\mu}$ could belong to a normally distributed population with population mean μ_0 and known standard deviation σ. In this case, the null hypothesis is H_0: $\mu = \mu_0$ and the alternative hypothesis is H_A: $\mu \neq \mu_0$. In a two-tailed test, H_0 is rejected when $\hat{\mu}$ is found to be outside the region of acceptance

$$\mu_0 \pm u_{1-\frac{1}{2}\alpha} \, (\sigma / \sqrt{n}) \qquad (1)$$

with $u_{1-\frac{1}{2}\alpha}$ being the appropriate quantile from the standard normal distribution. Suppose the sample was derived from a population with $\mu = \mu_A$ and equal standard deviation σ. An alternative test could then be $H_A : \mu = \mu_A$. The probability of rejecting H_0 when this H_A is true is the probability that $\hat{\mu}$ lies outside the interval (1) under H_A, which is

$$P\left(\underline{u} \leq \frac{(\mu_0 - \mu_A)}{(\sigma/\sqrt{n})} - u_{1-\frac{1}{2}\alpha}\right) + P\left(\underline{u} \geq \frac{(\mu_0 - \mu_A)}{(\sigma/\sqrt{n})} + u_{1-\frac{1}{2}\alpha}\right) \qquad (2)$$

with $\underline{u}$ being $(\hat{\mu} - \mu_A)/(\sigma/\sqrt{n})$, a standard normal variate. This probability is the power of the experiment $(1 - \beta)$ at $\mu = \mu_A$. It can be seen from this formula that the power depends on the significance level α, the absolute difference between μ_0 and μ_A, n, and σ. A formula for n, when a power of 1-ß is wanted at a difference of means of $\delta = \mu_A - \mu_0$ is

$$n = ((u_\beta - u_{1-\frac{1}{2}\alpha})^2\sigma^2) / \delta^2 \qquad (3)$$

N is proportionally related to the variance σ^2 and inversely related to δ. (N.B.: in a one-tailed test, $u_{1-\frac{1}{2}\alpha}$ becomes $u_{1-\alpha}$).

In most experiments, the situation is more complicated than in the example above. In many cases, two populations (for example, a control population and a population receiving a specific treatment) are to be compared, with equal but unknown σ^2. In such cases, the sample standard deviation *s* is used as estimate for σ. The *u*-values from the standard normal distribution are replaced by t-values from the t-distribution with (2n-2) degrees of freedom. However, a similar relation as (3) can be deduced for a one-tailed test:

$$n = 2\frac{\sigma^2}{\delta^2}\,(t_{2n-2;\beta} - t_{2n-2;1-\alpha})^2 \qquad (4)$$

Figure 5 is a graphical representation of equation 4.The relation between the sample standard deviation and the number of animals needed to obtain a significant treatment effect is depicted at a power of 0.8 and 0.9. In this example, μ_0 is assumed to be 2.0 and μ_A 1.8 and 1.6 ($\delta = 0.2$ resp. 0.4).

The example in figure 5 is derived from an experiment we conducted in our laboratory with common carp. Under normal circumstances, female common carp reach ovary weights of approx. 2 grams at 200 days of age. Lower ovary weights (one-tailed test) can be expected in carp treated with the antibiotic flumequin since this is known to reduce the rate of cell proliferation in carp (T. Van der Heijden, personal communication). We were able to detect a significant effect in an all-female F1 hybrid of common carp. In an all-female partly outbred strain, a similar reduction in gonadal development was observed. However, this effect was statistically not significant mainly due the higher standard deviation in this strain (fig. 6). The power of the t-tests could be calculated as 0.93 resp. 0.70.

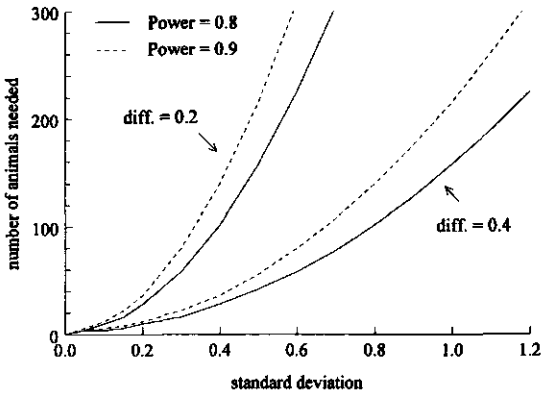

Figure 5. Relation between the standard deviation and the number of experimental animals needed for the comparison of two population means using a one-tailed t-test. Level of significance $\alpha = 0.05$; $\mu_0 = 2$. Standard deviations under H_0 and H_A are considered to be equal. Lines are drawn for tests with an expected difference of the means of 0.2 and 0.4, as well as for a test-power of 0.8 and 0.9.

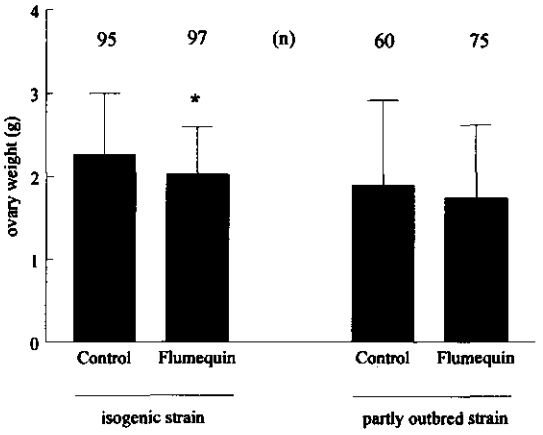

Figure 6: Reduction in ovarian weight at the age of 200 days in an isogenic strain and a partly outbred strain of common carp after oral administration of the antibiotic flumequin. The antibiotic was dissolved in fish oil and mixed with a commercial fish feed. The dose administered was 6 mg of antibiotic per kg body weight per day. The period of administration was between 28 and 42 days of age, i.e. the period of proliferation of the primordial germ cells. Control animals received untreated food (only fish oil added). (n) = number of fish ; * = sign. different ($P<0.05$); bars represent standard deviation.

Allocation of animals between and within inbred strains

When testing a panel of inbred strains in a factorial design, differences in main effects of the strains reflect the amount of (genetic) variation present within the experiment. In the ideal situation, this amount of genetic variation should reflect genetic variation in the outbred population, so generalization of test results would become possible. For reasons described later in this paper, this ideal situation is unlikely to be attained.

Below follow some considerations on the allocation of animals between and within inbred strains. Assume that a random sample of inbred strains from an infinite pool of inbred strains (with mean μ_s) is drawn. From each strain, an equal number of units is drawn on which trait $\underline{y}$ is measured. Assume that the total number of animals to be used (n) is fixed. A model for $\underline{y}$ is

$$\underline{y}_{ij} = \mu_s + \underline{a}_i + \underline{e}_{ij} \qquad (5)$$

with i = 1,...,I (the number of strains) and j = 1,...,J (the number of units (animals) within strains), $\underline{a}_i$ is the random effect of strain i with $\mathcal{E}(\underline{a}_i) = 0$ and var $(\underline{a}_i) = \sigma^2_a$, and $\underline{e}_{ij}$ is the residual error corresponding to animal j in strain i, with $\mathcal{E}(\underline{e}_{ij}) = 0$ and var $(\underline{e}_{ij}) = \sigma^2_e$. We see that $\mathcal{E}(\underline{y}_{ij}) = \mu_s$ and var $(\underline{y}_{ij})$ = var $(\underline{a}_i)$ + var $(\underline{e}_{ij}) = \sigma^2_a + \sigma^2_e$, the sum of the between strain and within strain variance, also called variance components. From the random sample, μ_s is estimated with the mean $\underline{y}..$, with variance

$$var\ (\underline{y}..) = \frac{\sigma_a^2}{I} + \frac{\sigma_e^2}{IJ} \qquad (6)$$

Optimal allocation of the fixed number (IJ) of experimental units within and between inbred strains will depend on the quantity to be estimated and possible constraints, for example with respect to costs. If the main interest lies in the mean μ_s, it is seen from (6) that the number of strains I should be as large as possible. So, n strains should be taken with only one unit per strain. This design will give an estimate of μ with minimum variance. However, no separate estimates of the variance components σ^2_a and σ^2_e are possible.

Often, it is more expensive to include more strains into the sample compared to including more units within strains. If such cost considerations are to be taken into account, the following may be shown with respect to optimal allocation of units within and between strains. Suppose one strain costs C_a (cost-units), compared to C for units within strains. So, the total (variable) cost $K = IC_a + IJC$. Suppose we would like to use a design, for a fixed amount of (variable) costs K, which minimizes var($\underline{y}..$). The minimum is achieved for

$$J = \left(\frac{\sigma_e}{\sigma_a}\right) \sqrt{\left(\frac{C_a}{C}\right)} \qquad (7)$$

and I follows by backsubstitution of J into $K = IC_a + IJC$. This formula shows that more units per strain should be taken if the within strain variance component gets larger compared to the between strain component. Also, if the cost per strain gets higher compared to cost within strain, more units per strain should be taken (Snedecor and Cochran, 1989).

Often, interest lies in the comparison of a treatment group with a control group. Suppose a factorial design is used which, per strain, allocates at random half of the J units to the treatment and half to the control. In fact, this is an ordinary randomized block design. The means in the treatment and control group are resp. μ_T and μ_C, which are estimated with the ordinary sample means. Assume no interactions between strains and treatment/control are present. The main interest is the difference $\mu_T - \mu_C$. The estimator of this difference has variance $4\sigma^2_e/IJ$, which does not depend on the between strain variance σ^2_a and the choice of I and J for fixed n. Because the variance components σ^2_a and σ^2_e are unknown, they have to be estimated from the data by (MSA-MSE)/J and MSE respectively, with MSA and MSE being the well-known mean squares. For the comparison of μ_T and μ_C only σ^2_e matters, as shown above. Most information on σ^2_e is obtained if a design is used with only 1 strain. However, such a design does not allow estimation of σ^2_a.

A reasonable choice may be to take I and J such, that both σ^2_a and σ^2_e are estimated as precise as possible. It may be shown that this is the case when

$$I = \frac{(n^2k + 2n)}{(nk + n + 1)} \qquad (8)$$

with k being the ratio σ^2_a / σ^2_e (Scheffé, 1959). As in formula (7), the ratio k plays a role. This implies that when σ^2_a and σ^2_e are not known, preliminary tests have to be performed to get an idea about the magnitude of k. Figure 7 gives a graphical representation of (8). It can be seen that when for a certain trait the within-strain variation is relatively small, many strains should be tested with few animals per strain. When between-strain variation is small, few strains with many animals per strain should be tested. N.B.: note that formulae (7) and (8) have to be used with care since I and J are integers and must divide the pre-fixed n.

In practice other factors such as availability of inbred strains, space in the laboratory and costs will play an important role. Still, this theoretical derivation for the number of inbred strains to

be tested is indicative, and might lead to a more efficient use of experimental animals. Haseman and Hoel (1979) compared the power of one-tailed Fisher's exact tests in single-strain vs multi-strain testing in a hypothetical treatment, inducing an increase in tumor frequencies at low levels, moderate levels or high levels. When subgroups (analogous to inbred strains) would be randomly taken from an outbred stock, control and treated animals would be tested within each subgroup. The total number of animals tested was equal in both tests. They found that the power of multi-strain testing exceeded that of single-strain testing when 3 or more inbred strains would have been used. However, Felton and Gaylor (1989) showed that an increase in power is only obtained when the chemical induces an increase in tumor frequency of 0.10 in one or more strains in the set (reviewed in Lovell, 1993).

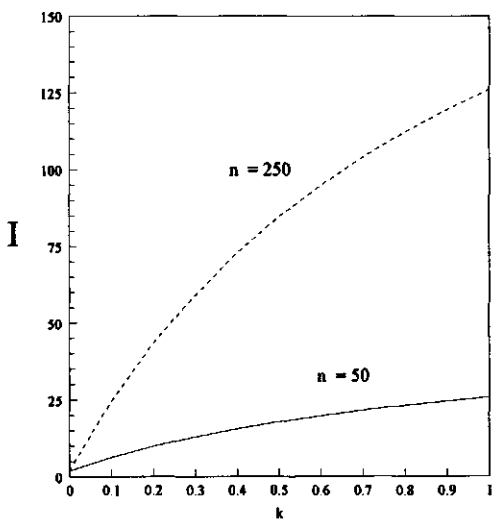

Figure 7: Illustration of the relation between the ratio between-strain : within-strain variation ($k = \sigma^2_s/\sigma^2_e$) and the number of inbred strains to be tested within one experiment (equation (8) in the text). Lines are drawn for the situation where the total number of animals used in the experiment (n) is 50 and 250.

Consequences of inbreeding on genetic variance

In any outbred population, the total variance (V_{tot}) can be partitioned in genetic variance (V_G), environmental variance (V_E) and the correlation between these two components (V_{GE}). Genetic variance in its turn can be further divided in an additive genetic component (V_A), a dominance component (V_D) and an interaction component (V_I) (Falconer and Mackay, 1996):

$$V_{tot} = V_G (= V_A + V_D + V_I) + V_{GE} + V_E$$

When conventionally inbreeding in a population (for example by full sib mating), several families will be established. During inbreeding, genetic variance between and within families will increase resp. decrease with the coefficient of inbreeding F (Falconer and Mackay, 1996):

$$V_{tot} = V_{G\text{-between families}} + V_{G\text{-within families}} = 2FV_G + (1-F)V_G = (1 + F)V_G$$

When inbreeding has been completed (F = 1), the population will consist of inbred strains with no genetic variance within strains. Using gynogenesis or androgenesis, fully inbred individuals are obtained in one single generation. In this situation the following formula applies:

$$V_{tot} = V_{G\text{-between families}} + V_{G\text{-within families}} = 2fV_G + (1 +F - 2f)V_G = (1 + F)V_G$$

where f = the coefficient of coancestry (= the chance that a random allele in individual X is identical by descent to a random allele in individual Y) among individuals of the same family (Falconer and Mackay, 1996). Homozygous gynogenetic individuals are fully inbred (F = 1) and the coefficient of coancestry f of individuals belonging to the same gynogenetic family is ½ (Bongers et al., 1996a). As a result, the genetic variance within families is equal to the genetic variance between families and the total amount of genetic variance after gynogenetic reproduction is doubled. This is however only valid when the founder males and females of homozygous families are fully outbred (Bongers et al., 1996a).

Implications for the use of inbred strains

Genetic variance in homozygous populations only consists of additive genetic variance. Dominance-effects are absent due to the absence of heterozygotes. Interaction-effects (e.g. additive x additive) are thought to be negligible (De Boer and Hoeschele, 1993).

Figure 8 demonstrates these consequences of inbreeding concerning distribution of genetic variance for conventional inbreeding and for inbreeding by gynogenesis or androgenesis. From this figure, it can be seen that when a panel of inbred strains is to be used for experimental purposes, gynogenetic or androgenetic inbred strains are to be selected from different families. Selecting strains from the same family is equal to testing within a genetic range of V_A, while selecting strains from different families equals testing within $2V_A$. When outbred strains are used, results obtained are representative for a range of genotypes with variance $V_A + V_D + V_I$. When F1 hybrids are produced from not related inbred strains (outbreeding, F = 0), results are also representative for $V_A + V_D + V_I$. Whether the absence of dominance or interaction effects in inbred strains will affect the outcome will depend on the trait under investigation.

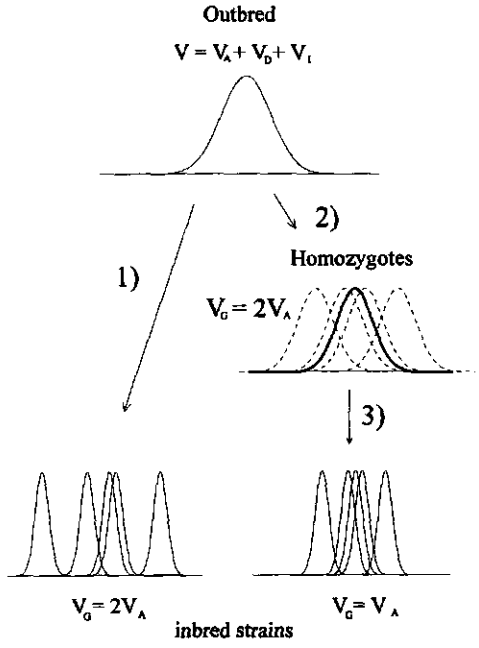

Figure 8: Consequences of inbreeding on genetic variance by conventional inbreeding (full sib-mating, 1) or by repeated gynogenesis or androgenesis (2,3). Starting-point is an outbred population with genetic variance $V_G = V_A$ (additive genetic variance) + V_D (dominance variance) + V_I (interaction variance). Through conventional inbreeding, separate families will be established with reduced within-family variance and increased between-family variance ($2V_A$). After gynogenetic or androgenetic reproduction of several parents (2), genetic variance becomes $2V_A$, equally divided within and between families (V_A). Within family variance is indicated by the solid line in 2). When producing inbred strains by repeated gynogenesis or androgenesis (3), genetic variation between lines remains V_A when homozygous parents come from the same gynogenetic family.

Residual variation within inbred strains

Phenotypic uniformity in inbred strains is depending on how much of the variance in outbreds is of genetic origin. For characteristics with high heritability (i.e., a large part of the phenotypic variance is genetic variance), inbred strains are likely to be more uniform than outbred strains. For characteristics with low heritability, this may not be the case. However, the increased susceptibility of inbred strains for environmental sources of variation can offset the reduced genetic variance, with an increase of the total phenotypic variance as a result (Festing, 1979; Falconer and Mackay, 1996). When homozygous parents are used in gynogenesis or androgenesis, the resulting homozygous clonal offsprings still display high residual variation and large amounts of deformed individuals (phenodeviants) (Komen et al., 1993; Taniguchi et al., 1994). Due to absence of genetic variance, only environmental variance (V_E) can be responsible for this. In a previous paper, we proposed three types of V_E (Bongers et al., 1996c):

- "true V_E", consisting of inter-individual variation. Homozygous populations are in general more susceptible to this source of V_E (Falconer and Mackay, 1996).
- intra-individual variability, or developmental instability (DI). Through reduced homeostasis of homozygous genotypes, the buffering of developmental processes against environmental and physiological sources of variability decreases, resulting in DI (Lerner, 1954; Palmer and Strobeck, 1986).
- V_E, generated from negative side effects of the chromosome manipulation treatment on (maternal) cytoplasmic components (Komen et al., 1993; Mair, 1993; Bongers et al., 1996c). This last source of V_E can be referred to as "embryonic damage", ED.

We found that ED is the main source of variation in gynogenetic and androgenetic offsprings. The level of inbreeding did not affect true V_E and DI (Bongers et al., 1996c). Table 1 summarizes observations of V_E for several characteristics in inbred and (partly) outbred strains of fish. The inbred fish strains in this table were either produced by gynogenetic or androgenetic reproduction of a homozygous parent, or by crossing (near) identical homozygous conspecifics. It is clear from this table that especially in carp, inbred strains, produced by chromosome manipulation do not display lower coefficients of variation than (partly) outbred strains. However, in most cases the (partly) outbred strains in this table can be considered more as inbred strains than outbred strains. For example, in reference a, c and e in table 1, "outbreds" were produced by crossing a homozygous gynogenetic female with an outbred male. Still, when inbred strains of fish are to be used, they should be derived from crossing homozygous clonal conspecifics (ref. a and b vs c and d !). Chromosome manipulation should only be used to generate homozygous broodstock.

F1 hybrids combine both genetic and phenotypic uniformity (Festing, 1979; Falconer and Mackay, 1996). Still, coefficients of variation in F1 hybrids of fish are still relatively large when

Table 1. Coefficients of variation (cv) of various characteristics in inbred and (partly) outbred fish strains. Inbred strains were produced by gynogenetic or androgenetic reproduction of a homozygous parent, or by crossing two (near) identical homozygous individuals.

Species (ref.)	character		cv-inbred	cv-outbred	remarks
Cyprinus carpio (a)	length:	24 weeks	18	7	inbred = gynogenetic inbred; outbred = partly outbred
		30 weeks	22	9	
	body weight:	24 weeks	42	21	
		30 weeks	55	29	
(b)	body weight:	10 weeks	31 - 64	15 - 25	inbred = 3 androgenetic inbred strains; outbred = partly outbred
(c)	length:	14 weeks	10	13	inbred = cross of 2 homozygotes, 99 % identical
	body weight:	14 weeks	27	32	
	# dorsal fin rays		4	7	
(d)	body weight:	3 weeks	27	31	inbred = cross of 2 homozygotes, 99 % identical
Plecoglossus altivelis	body weight:	6 months	11 - 12	22 - 27	inbred = gynogenetic inbred; outbred = partly outbred
(e)		9 months	12	18	
	length:	6 months	3	5 - 7	
		9 months	3	5	
	# dorsal fin rays		5	4	
(f)	hematocrite		12 - 16	17	inbred = cross of 2 identical homozygotes
	# red blood cells		15 - 21	16	
	hemoglobin (g/dl)		7 - 15	13	
	mean corpuscular volume		2 - 3	3	
	glucose (mg/dl)		3 - 17	30 - 46	

(a): Komen et al., 1993 (b): Bongers et al., 1995 (c): Bongers et al., 1996c (d): Bongers et al., unpubl. (e): Taniguchi et al., 1994 (f): Del Valle and Taniguchi, 1995

compared to inbred strains of laboratory rodents (table 2) and vary greatly between strains. According to Festing (1979), residual variation in mammals can be attributed to competition between animals, chance variation *in utero*, chance contamination by micro-organisms etc. For example, Gärtner (1990) analyzed environmental variation for quantitative traits in laboratory strains of mice and rats and determined that only 20 - 30 % of the random variation was true environmental variation. The remaining 70 - 80 % was attributed to a component of variation, effective at or before fertilization and could originate from ooplasmic differences between eggs. The fact that fish are poikilothermic make them more susceptible to similar sources of variation, resulting in a large phenotypic variance, compared to homeothermic animals (Allendorf et al., 1987).

Table 2. Coefficients of variation of body weight (BW) and (relative) testis weight (TW) in laboratory strains of rodents and common carp. TSI = testis somatic index: (testis weight/body weight) x 100 %.

Species (ref.)	character		cv	type (number) of strains
Mouse, *Mus musculus*				
(a)	TSI,	91 days	5 - 10 %	inbred (3)
(b)	BW,	30 days	10 - 25 %	inbred (3)
		60 days	9 - 11 %	
		120 days	8 - 10 %	
	TW,	30 days	15 - 24 %	
		60 days	9 - 15 %	
		120 days	9 - 16 %	
Rat, *Rattus novegicus*				
(c)	BW,	age ?	8 %	outbred (1)
	TW		6 - 8 %	
	TSI		5 - 8 %	
Golden Hamster, *Mesocricetus auratus*				
(d)	TW,	age ?	7 - 15 %	outbred (1)
Common carp, *Cyprinus carpio*				
(e)	TSI,	100 days	20 - 38 %	partly inbred (2)
			13 - 36 %	F1-hybrid (2)
	BW,	210 days	30 - 39 %	partly inbred (2)
			14 - 27 %	F1-hybrid (2)
(f)	BW,	168 days	21 %	partly inbred (1)
			15 - 23 %	F1-hybrid (2)
	BW,	210 days	29 %	partly inbred (1)
			11 - 16 %	F1-hybrid (2)
(g)	BW,	240 days	19 - 41 %	F1-isogenic (12)
	TW,	240 days	41 - 106 %	
	TSI,	240 days	26 - 73 %	

References: (a) Chubb, 1992; (b) Shukri and Shire, 1989; (c) Vaughan et al., 1988; (d) Jackson et al., 1984; (e) Bongers et al., unpublished results; (f) Komen et al., 1993 (g) Bongers et al., 1996d.

Conclusions

Fish are important laboratory animal models. Frequently, experimental fish with unknown genetic background and raising history are used. This leads to low repeatability between experiments. When outbred strains are used, high intrastrain variability might obscure treatment effects. Also, high levels of inbreeding may still have accumulated in the outbred strains. To improve the quality (replicability, repeatability and reproducability) of experimental research with fish, inbred strains need to be established. Chromosome manipulation techniques (gynogenesis and androgenesis) can facilitate this. The best approach in bioassays (e.g., toxicological screening) is to use a panel of inbred strains to enable extrapolation experimental results to an outbred population. This could decrease the number of animals used and increase the power of the experiments at the same time, due to lower intrastrain variability. However, gynogenetic or androgenetic reproduction results in embryonic damage, thereby increasing coefficients of variation. Therefore, inbreds strains for research purposes should be derived from crossing two identical genotypes, of which one is hormonally sex reversed. F1 hybrids can also be used. Gynogenesis and androgenesis should then only be applied to generate homozygous broodstock.

When comparing coefficients of variation of inbred and F1 hybrids of fish to inbred strains of laboratory rodents, coefficients of variation are higher in fish strains. This could be attributed to the fact that fish are poikilothermic animals. As a result, fish are more susceptible to environmental sources of variation. Nevertheless, using inbred fish lines will increase experimental quality and leads to a more efficient use of experimental animals.

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Chapter 3

Androgenesis in common carp (*Cyprinus carpio* L.) using UV-irradiation in a synthetic ovarian fluid and heat shocks.

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Abstract
Haploid androgenetic offspring (max. 53.9 %) were obtained after UV-irradiating eggs using dosages of 150-300 mJ/cm^2 (duration: 135 to 270 seconds at an intensity of 1.1 mW/cm^2). During irradiation, eggs were stirred in a synthetic ovarian fluid to assure a homogenous irradiation of all pronuclei, without activating the eggs. Androgenetic origin was checked using a recessive colour marker ("blond"). Absence of black fry (dominant trait) in the offspring confirmed inactivation of the female genome. Besides androgenetic haploids, few biparental diploids hatched at the optimal dose (250 mJ/cm^2). Diploidy could be restored by heat shocks (40.0 °C, 2 min) applied at different times after fertilization (26, 28 and 30 minutes). Yields of putative androgenetic diploids ranged from 7.2 - 18.3 % at the time of hatching. Also, survival of the larvae 24 days after hatching was high (78 - 89 % of the initial number). After testing the homozygous nature of the fish, they will be used to generate isogenic strains.

1. Introduction

In androgenesis, the female genome is inactivated. Duplication of the male genome can be achieved by suppression of the first cleavage. Androgenetic reproduction techniques can be used for gene banking and inbreeding. In our laboratory, we are aiming to use androgenetic strains of common carp to study sex determination (Komen et al., 1992a) and to develop inbred lines for research purposes. Thus far, androgenesis has been applied with relatively little success in amphibians and fishes (table 1).

Inactivation of the female genome can be done by gamma-rays (^{60}Co), X-rays, pressure shocks, chemicals and UV-irradiation. In gynogenesis, UV irradiation of paternal DNA is widely accepted as the method of choice since Chourrout (1984) demonstrated the presence of paternal chromosome fragments in homozygous gynogenetic diploid *O. mykiss*, when sperm was submitted to gamma-rays. Thorgaard et al. (1985) did similar observations in *O. mykiss*. Disney et al. (1987) showed that production of transgenic *O. mykiss* was possible after ^{60}Co-irradiation of brook trout (*Salvelinus fontinalis*) sperm. Carter et al. (1991) recently demonstrated by DNA-fingerprinting the transmission of paternal DNA in gynogenetic offspring of *O. aureus* after UV-irradiating sperm (19.8 mJ/cm^2). They also stated that incorporation of DNA might be expected to occur more readily when highly energetic gamma-rays are used because of induced DNA-fragmentation.

We started the production of haploid androgenetic offspring in common carp using UV-irradiation. Eggs were stirred in a synthetic ovarian fluid to assure a homogenous irradiation of all pronuclei and to minimize UV-damage to cytoplasmic constituents.

Table 1: Summary of methods used to eliminate the female genome and to restore diploidy in androgenesis research. When possible, hatching percentages are calculated as percentage of the total number of incubated eggs (rel %: expressed as % of hatching in the untreated control). References: 1 = Purdom, 1969; 2 = Arai et al., 1979; 3 = Gillespie and Armstrong, 1980; 4 = Gillespie and Armstrong, 1981; 5 = Briedis and Elinson, 1982; 6 = Parsons and Thorgaard, 1984; 7 = Parsons and Thorgaard, 1985; 8 = Scheerer et al., 1986; 9 = Thorgaard et al., 1990; 10 = Scheerer et al., 1991; 11 = May et al., 1988; 12 = Grunina et al., 1990; 13 = Bongers et al., present paper.

Species	Ref.	Inactivation of female genome; dose	Diploidization treatment	Genetic marker	Androgenetic yield	Remarks
Platichthys flesus	1	^{60}Co; 67 kR	cold shock	-	no diploids, ?% haploids	-
Oncorhynchus masou	2	^{60}Co; 50-60 kR	-	-	35% haploids at day 40 0% hatching	-
Ambystoma mexicanum	3	UV; 47 mJ/cm^2	pressure	colour	hatching: 2.4% diploids	hatching in one out of four trials;insufficient elimination of female genome
	4	UV; 40-60 mJ/cm^2	heat shock	colour	hatching: 0.7-4.6% diploids (% of cleaving eggs)	-
Rana pipiens	5	Pressure; 7000 psi, 6 min	-	isozymes morphology	70% haploids at tailbud-stage, ?% hatching	-
		D_2O-80%, 10 min.	-		50% haploids at tailbud-stage, ?% hatching	
Oncorhynchus mykiss	6	^{60}Co; 30 kR	-	colour	26.7% survival at day 20, 0% hatching	maternal DNA-fragments at high doses (50 kR)
	7	^{60}Co; 36 kR	pressure	colour	hatching: 32.5-38.9% diploids (rel.%)	

	8	^{60}Co; 36 kR	pressure	isozymes	hatching: 7.2-7.9% diploids (rel.%) feeding: 4.7-4.8% (rel. %) hatching: 8.8-9.5% diploids (rel. %) feeding: 5.6-6.8% (rel. %)	inbred sperm-source outbred sperm-source
	9	^{60}Co; 40 kR ^{60}Co; 40 kR ^{60}Co; 40 kR	pressure - pressure	-	hatching: 1.2% diploids, feeding: 0.6% hatching: 11.8% diploids, feeding: 10.3% hatching: 0.7% tetraploids, feeding: 0.3%	diploid sperm-source tetraploid sperm-source tetraploid sperm-source
	10	^{60}Co; 36 kR	pressure	isozymes, colour	hatching: 2.4-2.7% diploids feeding: 1.8-1.9% hatching: 1.5-1.6 % diploids feeding: 1.2-1.3 %	outbred sperm-source inbred sperm-source
Salvelinus fontinalis	11	^{60}Co; 88 kR	pressure	allozymes	37% diploids at eyed stage (rel. %) ? % hatching	-
Cyprinus carpio	12	X-ray; 25-30 kR	heat shock	colour	hatching: 9% diploids, expressed as% of survival after 24 hours of incubation (no heat shock: 12 % haploids)	insufficient elimination of female genome
	13	UV; 100-250 mJ/cm^2	heat shock	colour	hatching: 8.6-19.3 % diploids 24 days: 6.2-15.8 % (no heat shock: 52 % haploids)	incubation in ovarian fluid during irradiation

High yields of androgenetic diploids have been obtained in *O. mykiss* (Parsons and Thorgaard, 1985; Scheerer et al., 1986; Thorgaard et al., 1990) using gamma-rays to inactivate the female genome. DNA-fragmentation might have occured, although isozyme analysis revealed 100 % paternal inheritance. Diploidization was done using pressure shocks. Grunina et al. (1990) described a method to produce androgenetic common carp. However, significant numbers of biparental diploids among the androgenetic offspring were found, indicating insufficient inactivation of the female DNA by X-rays.

In our research, diploidization-inducing treatments consisted of heat shocks which yield consistent results in gynogenetic experiments (Komen et al., 1991). As a genetic marker, a coloration affecting mutation ("blond") was used to distinguish androgenetic from biparental offspring.

2. Material and Methods

Genetic markers

Male broodstock were homozygous for a recessive mutation causing absence of normal melanophore development (b1,b2/b1,b2) and therefore appear blond. Homozygous (B1,B2/B1,B2) and heterozygous (B1,B2/b1,b2) black clones of females were used to minimize variation in egg quality. Homozygous clones were used in experiments to inactivate the female genome. All hatched blond haploid individuals could thus be regarded as androgenetic. Heterozygous clones were used in experiments to restore diploidy after UV-irradiation. The punnet square, depicted in table 2, shows the expected frequency of blond animals after crossing a heterozygous female with a blond male. Absence of black fry in UV-irradiated groups confirms inactivation of the female genome. We have also tried to induce diploidy using eggs from homozygous females. However, heat shocking these eggs resulted repeatedly in complete mortality within 24 hours of incubation. All broodstock was maintained at 25 °C in 800 liter rectangular tanks with recirculating water and were fed carp pellets (25 % crude protein, Provimi b.v., Rotterdam, the Netherlands) 0.6 % of their body weight per day.

Irradiation, fertilization and heat shocking

Eggs and sperm were obtained as previously described (Komen et al., 1991). After stripping, eggs were kept at room temperature. Milt was diluted 1:3 with physiological saline (0.9 % NaCl) and stored on ice.

Table 2: Punnet square for possible genotypes regarding coloration in an offspring from a cross between a heterozygous black female (B1,B2/b1,b2) and a blond male (b1,b2/b1,b2). The expected frequency of blond animals among the offspring will be 25 %.

gametes: ♀:	B1,B2	B1,b2	b1,B2	b1,b2
♂: b1,b2	B1,b2/b1,B2	b1,b2/B1,b2	b1,B2/b1,b2	b1,b2/b1,b2

Three methods of irradiation were examined:

(1) Dry method: Egg-samples were gently dispersed to obtain a single layer of eggs, sticking to the surface of the petri-dish.

(2) Manual stirring method: Egg-samples were put in a petridish and 5 ml of a synthetic ovarian fluid (OF) was added to obtain a single layer of eggs, floating in the fluid. OF consisted of: 4.11 gr BSA fraction IV per litre, 3.8 mMOL Na_2HPO_4, 118.0 mMOL NaCL, 12.7 mMOL KCl, 0.7 mMOL $MgCl_2*6H_2O$, 2.7 mMOL $CaCl_2$, 5.5 mMOL Tyrosin and 5.5 mMOL Glycin in distilled water (after Plouidy and Billard, 1982 and Epler et al., 1984). The pH was adjusted to 8.14. Osmolarity was 262 mOsm.

In this ovarian fluid, eggs did not become adhesive and were not activated. During gentle manual stirring, the eggs were able to roll in the fluid.

(3) Mechanical stirring method: Instead of manual stirring, the petridish was placed on a mechanical stirrer (Schüttler MTS 2) after adding ovarian fluid to the egg sample.

Irradiation was done using a VL-6 UV-lamp (7.5 W, 254 nm) in a UV-CN6 darkroom (Ankersmit, the Netherlands). The total (cumulative) energy (mJ/cm^2) was recorded using a VLX-3W sensor (Ankersmit, the Netherlands), which was positioned at the same (fixed) distance from the UV-source as the eggs. The intensity was kept constant at 1.1 mW/cm^2.

After irradiation, egg samples were immediately mixed with 0.25 ml of sperm suspension and fertilized by adding water (24 °C). They were transferred to baskets (diam. 10 cm.) with a mesh-bottom (mesh-size 0.5 mm) and incubated at 24 °C in the dark.

Heat shocks were administered by transferring the eggs from the incubation-system to a waterbath of 40.0 °C for 2 minutes.

Experiments

Experiment 1 was done to inactivate the female genome. In this experiment, the dry method and the manual stirring method were compared. Total UV-dosages tested ranged from 0 to 104 mJ/cm^2, which equals irradiation-durations of 0 to 95 seconds.

Experiment 2 was conducted to standardize the manual stirring procedure of experiment 1 by mechanically stirring the eggs during irradiation. The range of total UV-dosages tested was increased from 0 to 500 mJ/cm^2, which equals irradiation-durations of 0 to 455 seconds respectively.

Experiment 3 was undertaken to duplicate the male genome after inactivation of the female genome. Heat shocks were administered at 26, 28 or 30 minutes after fertilization. Eggs were irradiated with a total UV-dose of 100, 150, 200 or 250 mJ/cm^2 to test for interaction between irradiation-dose and heatshocking on mortality. All hatched androgenetic diploids, produced within the same UV-dose treatment were pooled and subsequently raised at 25 °C. The resulting 4 groups of fry were fed freshly harvested *Artemia* nauplii ad libitum during the first 24 days after hatching.

A survey of all the controls and treatments are presented in table 3. To check the quality of eggs, yields of normal fry from non-irradiated eggs fertilized with milt were recorded. In experiment 2 and 3, this control (Co) was carried out at the start (Co-start) ànd at the end (Co-end) of the experiment to estimate the decrease in egg quality during the experiment. To determine the effect of the ovarian fluid on yields of normal fry, eggs were incubated for 2 minutes in ovarian fluid (without irradiation) prior to fertilization (Ovarian Fluid control: OF). To determine the effect of stirring the eggs, a "mechanical stirring control" (MS) was introduced in experiment 2 and 3: eggs were mechanically stirred in ovarian fluid, without irradiation. In experiment 3, the efficiency of the UV-irradiation (UV-Control: UV-Co) was estimated by incubating non-shocked irradiated eggs, fertilized with milt.

Parameters recorded and statistical analysis.

Egg samples (controls and treatments) consisted of 150-200 eggs and were carried out in duplicate. At 96 hours after fertilization, the percentage of haploid androgenetic larvae, diploid androgenetic larvae, diploid biparental larvae and non-developed eggs were recorded. All percentages were expressed as percentage of the total number of incubated (treated) eggs.

In experiment 1 and 2, yields of haploid androgenetic larvae and biparental larvae were compared using Duncan's multiple range test ($P < 0.05$) after arcsin transformation. In experiment 3, this procedure was followed concerning blond (putative androgenetic) and black biparental larvae. Additionaly, Yates' corrected chi-square for goodness of fit ($P < 0.05$) was used to determine if

the ratio black offspring : blond offspring in heat shocked treatments deviated significantly from the control (expected 3 : 1, see table 2).

Relative survival of the 4 groups of androgenetic diploids was determined per group at day 24 after hatching and calculated as (total number day 24 / total number at hatching) * 100 %. All analyses were carried out using BMDP computer programs (Dixon et al., 1988).

Table 3: Survey of egg treatment procedures and controls. Numbers between brackets refer to experiments. Further explanation: see text.

	incubation in ovarian fluid	stirring	UV-irradiation	heat shock
egg quality control at start (Co-start; 1,2,3)	no	no	no	no
egg quality control at end (Co-end; 2,3)	no	no	no	no
Ovarian Fluid control (OF: 1,2,3)	yes	no	no	no
Mechanical stirring control (MS; 2,3)	yes	yes	no	no
UV-control (UV-Co; 3)	yes	yes	yes	no
Treated eggs (1)	yes/no	yes/no	yes	no
Treated eggs (2)	yes	yes	yes	no
Treated eggs (3)	yes	yes	yes	yes

3. Results

Inactivation of the female genome

Experiment 1. The control for egg quality and the ovarian fluid control yielded 61.3 % and 82.7 % normal diploids respectively.

Dry method (fig. 1A): Maximum percentages of 11.2 - 20.0 % of androgenetic haploids were obtained in the UV-dose range of 24 - 56 mJ/cm^2. At all dosages tested more biparental diploids than androgenetic haploids hatched.

Manual stirring method (fig. 1B): Maximum percentages of 40.4 - 56.6 % androgenetic haploids hatched in the UV-dose range of 40 -104 mJ/cm^2. Few eggs escaped irradiation, as can be concluded from the less than 1 % hatched biparental diploids in this range.

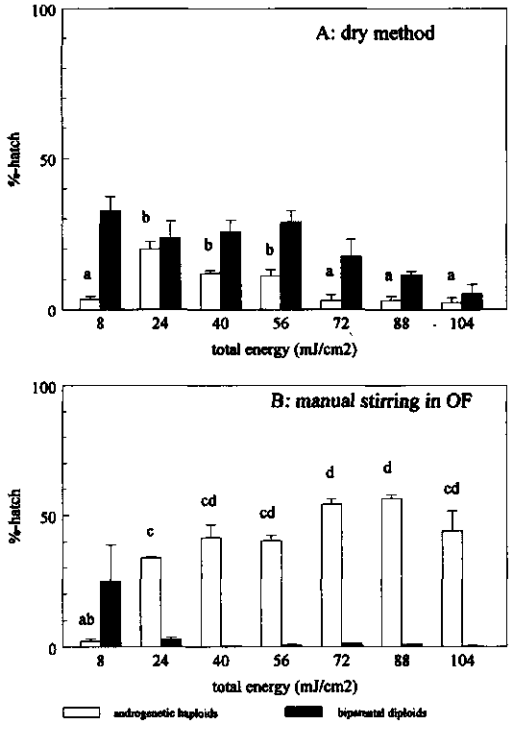

Fig. 1: Relation between the UV-dose administered (mJ/cm^2) and hatching of androgenetic haploid and biparental larvae using the dry method (graph A) and the manual stirring in Ovarian Fluid (OF) method (graph B). Groups with a common superscript do not differ significantly ($P<0.05$).

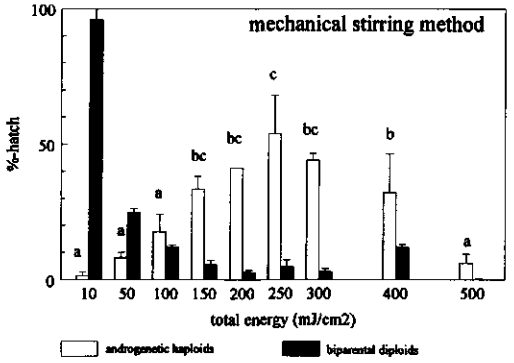

Fig. 2: Relation between the cumulative UV-dose administered (mJ/cm^2) and hatching of androgenetic haploid and biparental larvae using the mechanical stirring method. Groups with a common superscript do not differ significantly ($P<0.05$).

Experiment 2. In the start-control for egg quality, 90.6 % normal larvae hatched. In the end-control, this was only 28.1 % indicating a decrease in egg quality. The ovarian fluid control and the mechanical stirring control yielded resp. 90.5 % and 88.9 % normal diploids.
High percentages of androgenetic haploids hatched in the UV-dose range of 150 - 300 mJ/cm^2 (fig. 2). A peak of 53.9 % was recorded at a UV-dose of 250 mJ/cm^2, although not significantly different from the UV-dosages 150, 200 and 300 mW/cm^2. In the optimal UV-dose range, 2.7 - 5.5 % biparental diploids hatched.

Duplication of the male genome

Experiment 3. (table 4) In the start-control for egg quality, 95.5 % normal larvae hatched. In the end-control, this was 93.0 % indicating a constant egg quality during the experiment. The ovarian fluid control yielded 86.2 % normal larvae. Mechanical stirring did not affect hatching (96.6 % normal larvae). Hatching percentages of androgenetic haploids in the UV-controls (not shown in table 4) were 18.3 %, 9.9 %, 21.3 % and 16.3 % at the resp. dosages of 100, 150, 200 and 250 mJ/cm^2. The low proportion of blond diploid larvae in the UV-controls (10 - 24 %, see table 4) suggests absence of androgenetic diploids among these groups (see also table 2).

Table 4. Hatching percentages (expressed as percentage of total number of eggs incubated) ± s.d. of blond diploid larvae in relation to the total dose of UV (mJ/cm²) administered and the onset of the heat shock (40.0 °C, 2 min.).

	shock-onset (minutes after fertilization)			
UV-dose	26	28	30	UV-Co
100	11.5 ± 0.9^{bc} (86)	7.2 ± 0.5^{ab} (84)	9.1 ± 1.2^{abc} (100)	0.2 ± 0.6^{a} (10)
150	17.3 ± 0.2^{bc} (90)	12.9 ± 3.0^{bc} (93)	9.5 ± 2.5^{abc} (100)	1.3 ± 2.5^{a} (22)
200	8.4 ± 1.6^{abc} (91)	9.4 ± 1.6^{abc} (97)	11.5 ± 2.3^{bc} (100)	1.0 ± 2.4^{a} (24)
250	17.9 ± 2.4^{c} (100)	11.6 ± 1.0^{bc} (100)	18.3 ± 9.9^{c} (100)	0.4 ± 0.9^{a} (12)

Figures between brackets refer to the percentage of blond animals among the offspring. Treatments with a common superscript do not differ significantly ($P < 0.05$).
Hatching percentages of untreated eggs Co-start: 95.5 ± 0.2 (30); Co-end: 93.0 ± 4.7 (29); OF: 86.2 ± 1.7 (30); MS: 96.6 ± 0.2 (26). For abbreviations, see table 3.

Table 5: Survival of androgenetic diploids, obtained from experiment 3 after 24 days of feeding. Animals, produced within the same UV-treatment (100, 150, 200 or 250 mJ/cm²) were pooled and subsequently raised. Survival is calculated as (number at day 24/number at hatch) * 100 %.

UV-dose	n-hatch	n-day 24	%-survival
100	113	88	78
150	164	146	89
200	103	85	83
250	161	143	89

It can be concluded that androgenetic diploids can be produced after irradiation with varying dosages of UV at different times after fertilization. Figure 3 shows more detailed information on the hatching percentages of androgenetic and biparental diploids. In all irradiated and heat shocked treatments, the ratio black larvae : blond larvae in the offspring differed significantly from the ratio observed in the control ($P < 0.001$). However, only in the highest UV-dose (250 mJ/cm²) no black (biparental) larvae hatched (fig. 3B), probably indicating a production of 100 % androgenetic diploids. This was also the case in offspring, derived from treatments with shocks administered 30 minutes after fertilization.

Table 5 shows the survival of androgenetic diploids at day 24 after hatching. Relative survival percentages ranged from 78 to 89 %, demonstrating the viablility of androgenetic diploids.

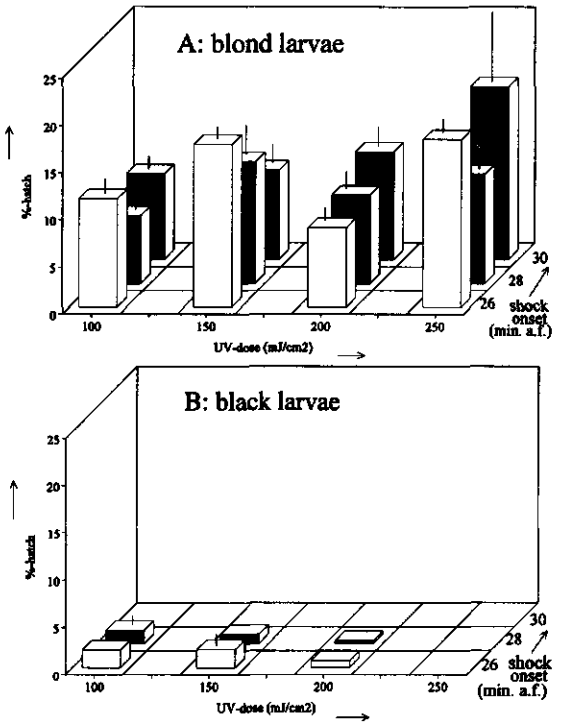

Fig 3: Hatching of blond larvae (A) and black larvae (B) after inactivation of the female genome by UV-irradiation (100, 150, 200 or 250 mJ/cm^2) and duplication of the male genome by heat shocking eggs (40 °C, 2 min. at 26, 28 or 30 minutes after fertilization).

Treatment effects

In all experiments, egg quality at the start of the experiments was high (61.3 %, 90.5 % and 95.5 % normal larvae hatched in resp. exp. 1, 2 and 3). Adding ovarian fluid did not affect hatching (OF: resp. 82.7 %, 90.5 % and 86.2 % normal larvae). During experiment 2, egg quality decreased whereas this was not the case in experiment 3 (Co-end: resp. 28.1 % and 93.0 % normal larvae). Stirring eggs seemed to be of no effect (MS: resp. 88.9 and 96.6 % normal larvae in experiment 2 and 3). Eggs in experiment 2 were less susceptible to UV-irradiation than in experiment 3 (UV-control: resp. 52 % and 9.9 - 21.3 % androgenetic haploids). By comparing the UV-control and the heatshocked eggs, it can be concluded that the eggs used in experiment 3 were highly resistant against the negative side-effects of the heatshock treatment (7.2 - 18.3 % androgenetic diploids).

4. Discussion

High yields of androgenetic haploids were obtained using a novel method which consists of stirring eggs in a synthetic ovarian fluid during UV-irradiation. Subsequent duplication of the male genome was done by heat shocks. Grunina et al. (1990) reported a maximum of 12 % hatched androgenetic haploids in common carp after irradiation with X-rays (dose: 25 - 30 kR). Hardly any biparental diploids hatched at this X-ray dose. With our method, we could increase hatching of androgenetic haploids up to 53.9 % (exp.2). Eggs were irradiated with a UV-dose of 250 mJ/cm^2. Using the dry method, only the nuclei that were oriented towards the UV-lamp were able to receive the correct UV-dose, resulting in low survival of androgenetic haploids and high survival of biparental diploids (exp. 1). The adding of a synthetic ovarian fluid facilitated homogenous irradiation of all nuclei.

Grunina et al. (1990) produced androgenetic diploids after application of a heat shock (40.5-41 °C, 2-3 min.) with persisting amounts of biparental diploids among androgenetic offspring. Exact figures on survival of androgenetic diploid larvae after hatching and subsequent feeding were not given. We produced 7.2 - 18.3 % putative androgenetic diploids after application of a heat shock (40 °C, 2 min.) at 26, 28 or 30 minutes after fertilization (exp. 3). Parsons and Thorgaard (1985) and Scheerer et al. (1986, 1991) reported complete absence of biparental diploids after ^{60}Co-irradiation in combination with pressure-shocks in *O. mykiss*. Our results show that high yields of androgenetic diploids can be obtained using a relatively simple method (UV-irradiation followed by a heat shock).

We used males, obtained after crossing an outbred male with a F1 hybrid female. F1 hybrids are essentially free of recessive lethal genes (Komen et al., 1991). As a result, the males carry less recessive alleles compared to outbred males. This might be the cause of our good results since Komen et al. (1992b) demonstrated that homozygous male parents yielded significantly more normal and fewer deformed fry than heterozygous males. However, Scheerer et al. (1986, 1991) could not improve hatching percentages of androgenetic diploids of *O. mykiss* when an inbred sperm-source was used (table 1). Egg quality alone seems to be the determining factor in the success of androgenesis experiments. In our laboratory, gynogenetic reproduction using homozygous broodstock yields consistently lower percentages of hatched diploids compared to experiments in which F1 hybrid broodstock is used (Komen et al., in prep.). Furthermore, great differences exist between egg batches in their susceptibility to the various treatment-components (compare f.e. exp. 2 with the UV-control in exp. 3). Hatching of normal larvae in the mechanical stirring control suggests that stirring is not harmful to eggs. However, in other experiments (data not shown) survival in the mechanical stirring control was greatly reduced. A methodological

approach, using appropriate controls seems to be essential to determine the separate effects of the various treatment components. We have experienced that only a minority of egg batches is resistant to the negative side effects of the total treatment, resulting in successful androgenesis. We used a colour marker to confirm the genetic origin of the offspring. At the optimal UV-dose to inactivate the female genome, few black (biparental) diploids survived (exp. 2). Gillespie and Armstrong (1980) used UV-irradiation to produce androgenetic *Ambystoma mexicanum*. They also found biparental diploids after irradiating eggs with a UV-dose of 47 mJ/cm^2. In experiment 3 (duplication of the male genome after inactivation of the female genome), we found black diploids at sub-optimal UV-dosages (< 250 mJ/cm^2), shocked at 26 or 28 minutes after fertilization. This implies that the blond offspring, produced with these treatments is not 100 % androgenetic since the female used in this experiment was heterozygous for the coloration-affecting mutation. Only the complete absence of biparental offspring (UV-dose: 250 mJ/cm^2 or shock-onset: 30 minutes after fertilization) strongly suggests the absence of the female genome. Still, it is not yet clear whether the genetic contribution of the female is fully eliminated, since (maternal) mitochondrial DNA and messenger-RNA are present in large quantities in eggs (Gardner et al., 1991), as well as possible maternal nuclear DNA-fragments (Carter et al., 1991). These last authors induced transmission of paternal DNA in gynogenetic *O. aureus* using UV-irradiation. The total UV-dose administered was 19.8 mJ/cm^2, which is less than 10 % of the UV-dose we found to be optimal in eliminating the female genome in carp.

Clonal nature of androgenetic offspring using a homozygous sperm donor will be tested in the near future using DNA-fingerprinting (Carter et al., 1991) or skin transplantation (Komen et al., 1990). Residual phenotypical variation within clonal lines might then be contributed to maternally derived variation.

Komen et al. (1991) stated that heat shocking eggs at the metaphase of the first mitotic division results in duplication of the genome in gynogenetic common carp. When applied earlier (prometaphase), the efficiency of the heat shock is reduced, resulting in high hatching percentages of haploid larvae. Heat shocking eggs, carrying a biparental genome at prometaphase might then result in the survival of biparental diploids. When shocked at metaphase these biparental genomes are duplicated. The resulting tetraploid fry is assumed not to be viable (Komen et al., 1991). In experiment 3, black (biparental) larvae were observed in treatments, shocked at 26 or 28 minutes after fertilization. No black larvae were observed in treatments, shocked at 30 minutes after fertilization, which supports the findings of Komen et al., (1991).

In the near future, sex ratios of androgenetic offsprings will become available. This will elucidate the question whether viable YY (homozygous) males can be produced through androgenesis. If viable, such males will be used to produce all-male isogenic offspring. All-female isogenic

offspring was already produced by Komen et al. (1991). The advantages of isogenic strains over outbred strains are obvious: due to little variation between the experimental animals, their number can be reduced. Isogenic strains can also be used for gene mapping and gene linkage studies (Taylor, 1978). In conclusion, isogenic strains could be of great value in standardization of experimental research.

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Chapter 4

Viable androgenetic YY genotypes of common carp, *Cyprinus carpio* L.

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Abstract
To elucidate the genetics of sex determination in common carp, *Cyprinus carpio*, we attempted to produce homozygous YY individuals using androgenetic reproduction techniques. XY-sperm donors were selected from a congenic strain with stable 50 : 50 sex ratios, produced by repeatedly backcrossing male offspring to a female from a homozygous clone. The YY-nature of androgenetic males was tested after crossing with females from different genetic backgrounds. One androgenetic male sired all-male offsprings indicating viability of the YY-genotype in common carp. Androgenetic reproduction of the same male yielded an all-male clone. Viability of the YY genotype in common carp is discussed from an evolutionary background.

Introduction

In higher vertebrates, the genetic sex is generally determined through structural distinct sex chromosomes. In mammals, females are the homogametic sex (XX) whereas males are heterogametic (XY). The sex determining region on the Y chromosome (SRY) has been identified (Goodfellow and Lovell-Badge, 1993). In reptiles, heteromorphic sex chromosomes are less common than in mammals and birds (Bull, 1980). In amphibia and fish, only in a minority of species morphological distinct sex chromosomes have been found, including male or female heterogamety and multiple sex chromosome patterns (Bull, 1980; Yamazaki, 1983; Chourrout, 1988). In fish, repetitive DNA-sequences, associated with sex chromosomes have only been found in *Xiphophorus* sp. (Schartl, 1988), *Oncorhynchus tschawytscha* (Devlin et al., 1991), *Poecilia* sp. (Nanda et al., 1990; 1993) and in *Leporinus elongatus* (Nakayima et al., 1994). Nevertheless, genetic sex determining systems have been shown to exist in numerous species (Hunter and Donaldson, 1983; Chourrout, 1988). Genetic sex determination is thought to be polygenic, with major sex determining genes located on possible sex chromosomes and minor genes located on autosomes (Hunter and Donaldson, 1983). In few species, sex differentiation can be influenced by environmental cues like temperature (Sullivan and Schultz, 1986; Mair et al., 1991; Beamish, 1992; Lagomarsino and Conover, 1993). However, these influences are less pronounced as in several reptiles, where f.i. a temperature difference of a few centigrades during egg-incubation can result in either all-male or all-female offsprings (Spotila et al., 1994).
Gynogenetic reproduction techniques and mating of sex reversed individuals have facilitated the rapid identification of sex determining mechanisms in several fish species (Chourrout, 1988). In our laboratory, we are interested in sex determination and sex differentiation in common carp, *Cyprinus carpio* for the production of standardized strains. Common carp is thought to have evolved from allotetraploidization (species hybridization) and many loci are still expressed in duplicate (Larhammer and Risinger, 1994). Although morphological distinct sex chromosomes cannot be identified (Kirpichnikov, 1981), sex determination is thought to be of the XX/XY-system. Conventional diploid offspring yields 50 : 50 sex ratios (Manzoor Ali and Satyanarayana Rao, 1989; Komen et al., 1992; Cherfas et al., 1994). Genetical females can be masculinized by

the administration of 17α-methyltestosterone (Nagy et al., 1981; Komen et al., 1989) and crossing these sex-reversed XX males with normal females yields all female offspring (Nagy et al., 1981). In most cases, gynogenesis leads to all-female offspring (Gomelsky et al., 1994). However, Komen et al. (1992) found an autosomal influence on sex differentiation in homozygous gynogenetic carp. In few cases, gynogenesis yielded females and males or intersexes in equal amounts. It was concluded that this was due to a mutant recessive minor sex determining gene named *mas-1*, which in homozygous condition gave rise to a male or intersex gonad. This indicated a ZZ/ZW sex determining system, with males or intersexes being *mas-1/mas-1* (≈ ZZ) and females being *mas-1/mas-+* (≈ ZW). Grunina et al. (1995) produced androgenetic male common carp. They reported that some androgenetic males reached maturity and sired all male offspring in normal crosses, indicating a YY male genotype. Sex ratios were not presented and conclusions regarding the sex determining system are not possible. YY individuals have been produced in various other species after crossing normal (XY) males with hormonally sex-reversed (XY) females (e.g. *Oryzias latipes* (Yamamoto, 1955; 1964), *Carassius auratus* (Yamamoto, 1975), *Oncorhynchus mykiss* (Johnstone et al., 1979; Chevassus et al., 1988), *Oncorhynchus kisutch* (Hunter et al., 1982), *Oreochromis niloticus* (Scott et al., 1989), *Ictalurus punctatus* (Davis et al., 1991) and *Poecilia reticulata* (Kavumpurath and Pandian, 1993). Feminization of male common carp using estrogens to obtain XY-females has yielded little or no succes (Komen et al., 1989; Bongers et al., 1991). As a result, androgenesis is the only method to produce YY males in this species. Recently, we succeeded in optimizing androgenesis in common carp using UV-irradiation to destroy the female nuclear material and heat shocks to restore diploidy (Bongers et al., 1994). Androgenesis in common carp is expected to result in 50 % homozygous XX females and 50 % homozygous YY males. To elucidate the genetics of sex determination in common carp, we attempted to produce homozygous YY individuals. We selected XY-sperm donors from a congenic inbred strain with stable 50 : 50 sex ratios. Sex ratios in the androgenetic offsprings were analysed. The YY-nature of androgenetic males was tested after crossing with females from different genetic backgrounds.

Material and methods

Artificial reproductions

All broodstock were maintained at 25 °C in 140 liter aquaria with recirculating water and were fed carp pellets (45 % crude protein, Provimi b.v., Rotterdam, the Netherlands) at 0.9 % of their body weight per day. Eggs and sperm were obtained as previously described (Komen et al., 1991). After stripping, eggs were kept at room temperature. Milt was diluted 1:3 with

physiological saline (0.9 % NaCl) and stored on ice. Normal fertilizations were performed by mixing 200-300 eggs with 200 µl of the sperm suspension and activated by adding water (24 °C). Androgenetic reproduction was done according to the method of Bongers et al. (1994). In short, egg-samples were put in a petridish and 5 ml of a synthetic ovarian fluid was added. The petridish was then placed on a mechanical stirrer and eggs were irradiated while gently stirring, facilitating the eggs to roll over in the ovarian fluid. The UV-intensity was kept constant at 1.1 mW/cm^2. Eggs were irradiated with a total UV-dose of 175 mJ/cm^2. Heat shocks were administered by transferring the eggs from the incubation-system (24 °C) to a waterbath of 40.0 °C for 2 minutes, 30 minutes after fertilization. This timing corresponds to the metaphase of the first mitotic division of the zygotes (Komen et al., 1991; Bongers et al., 1994).

Experimental design

A *X/Y* congenic homozygous inbred strain was produced by repeatedly backcrossing male offspring to a female from a homozygous clone (named WAUWG/E4, formerly called E4: Komen et al., 1991; 1992). Gynogenetic reproduction of these E4-females has yielded repeatedly all-female clones, indicating a stable female genotype of E4 (Komen et al., 1992; Komen and Bongers, unpublished results). Sex ratios were determined in each consecutive backcross (E4-*X/Y*-1 to E4-*X/Y*-6) and tested against the expected 50 : 50 sex ratio.

In a first attempt, androgenetic offsprings were produced from E4-*X/Y*-3 backcross progeny. Four males were used as sperm donor. A female from the E4-*X/Y*-1 cross served as egg donor. Control crosses (normal fertilizations) were also performed. Due to poor results (high percentages of sterile offspring, see results) a second attempt was made using three E4-*X/Y*-6 males. This time, outbred females served as egg donors. As a further control, one E4-*X/Y*-6 male was crossed to female E4 to produce E4-*X/Y*-7. In all offsprings, sex ratios were tested against the expected 50 : 50 sex ratio (XX : YY in androgenetics, XX : XY in controls).

We tested the presumed YY genotype of androgenetic males by crossing an androgenetic male from an E4-*X/Y*-3 sperm donor with females from different genetic backgrounds (female E4 and females K31 and A, originating from Israeli strains). The androgenetic male was also reproduced androgenetically to produce a putative YY-clone using an outbred female as egg donor.

Genetic markers

Males selected from backcross progenies were heterozygous for the dominant (wild-type) allele S and were thus fully scaled (*S/s*). In androgenetic reproductions, females used were also scaled (*S/s*). Offspring from normal fertilizations are then expected to consist of 75 % scaled carps and

25 % mirror carps. Androgenetic reproduction will result in 50 % scaled (*S/S*) and 50 % (*s/s*) mirror individuals. Hence, ratios of scaled and mirror individuals in androgenetic offsprings can be used to verify the homozygous nature of the offspring.

Experimental procedure and determination of gonadal sex

During the first four weeks after hatching, larvae were raised in 25 l aquaria at 25.0 °C. They were fed freshly hatched Artemia nauplii *ad libitum*. After this period, larvae were moved to another recirculation system with 70 l aquaria (T: 25.0 °C). They were fed vitamin C-enriched trout pellets at a daily ration of 25 gr per $kg^{0.8}$. Densities did not exceed 50 fish per aquarium.

At the age of 6 months, all offsprings were dissected and gonadal sex was determined macroscopically. At this age, males contain a well-developed testis with mature spermatozoa. Female gonads are still relatively undeveloped and contain only previtellogenic oocytes. Gonads containing both types of tissue were scored as intersex. Animals containing filiform gonads at this age are considered to be sterile (Komen et al., 1989).

All statistical analyses were done using Yates' corrected χ^2-test (BMDP computer programs, Dixon et al., 1988). P-values < 0.05 are regarded significant.

Table 1: Numbers of animals analysed (*n*) and percentages of male, female, intersex and sterile individuals in the congenic E4-*X/Y* strains, produced after repeatedly (n = 1 to 6) backcrossing male offspring to a homozygous female E4. Sex ratios were analyzed for deviation from a 50 : 50 sexratio (Yates' corrected χ^2 and P-value). In all cases, intersex and sterile individuals were left out from the analysis.

Cross	n	%-males	%-females	%-intersex	%-steriles	χ^2	P
E4-*X/Y*-1	44	54,5	45,5	0	0	0.05	0.83
E4-*X/Y*-2	35	42,9	22,9	11,4	22,9	0.58	0.45
E4-*X/Y*-3	91	48,4	50,5	0	1,1	0.00	1.00
E4-*X/Y*-4	70	47,1	44,3	0	8,6	0.00	1.00
E4-*X/Y*-5	119	45,4	38,7	0	16,0	0.18	0.67
E4-*X/Y*-6	55	52,7	43,6	0	3,6	0.09	0.77

Results

Sex ratios of the repeated backcrossings to produce the congenic strain are summarized in table 1. In cross E4-*X/Y*-2, intersexes were found whereas these were absent in other crosses. In crosses E4-*X/Y*-2 and E4-*X/Y*-5, large numbers of sterile animals were observed. However, in all crosses equal numbers of males and females were found. It can be concluded that sex ratios did not change during inbreeding and that autosomal influences affecting sex differentiation are absent in this pedigree.

Sex ratios of the four control and androgenetic offsprings produced from E4-*X/Y*-3 sperm sources are summarized in table 2. Although equal numbers of males and females were found in all crosses, large numbers of sterile individuals, especially in androgenetic offsprings, hampered the analysis. In the controls, the amount of sterile individuals ranged from 37,5 - 55,6 % and in the androgenetics from 70,6 - 94,4 %. Percentages of scaled individuals (not shown in table 2) ranged from 64 - 89 % in the controls and from 44 - 61 % in the androgenetic offsprings. This was not significantly different from the expected 75 % resp. 50 %, confirming the androgenetic nature of the offspring.

Table 2: Numbers of animals analysed (n) and percentages of male, female and sterile individuals after crossing 4 males from the E4-*X/Y*-3 offspring to an E4-*X/Y*-1 female (controls) and after androgenetic reproduction of these 4 males using eggs from the same female. All sex ratios were tested against the expected 50 : 50 sex ratio (XX : XY in controls; XX : YY in androgenetic offspring) using Yates' corrected χ^2. Sterile individuals were left out of the analysis.

Cross	n	%-males	%-females	%-steriles	χ^2	P
Controls:						
E4-*X/Y*-3-1	26	23,1	38,5	38,5	0,13	0,72
E4-*X/Y*-3-2	27	40,7	11,1	48,1	1,40	0,23
E4-*X/Y*-3-3	24	20,8	41,7	37,5	0,33	0,56
E4-*X/Y*-3-4	18	27,8	16,7	55,6	0,00	1,00
Androgenetics:						
E4-*X/Y*-3-1	18	0	5,6	94,4	0,00	1,00
E4-*X/Y*-3-2	34	2,9	26,5	70,6	2,14	0,14
E4-*X/Y*-3-3	16	0	6,3	93,7	0,00	1,00
E4-*X/Y*-3-4	15	20,0	0	80,0	0,37	0,55

Sex ratios in the androgenetic offsprings of 3 E4-*X/Y*-6 males contained 49,0 - 53,2 % males and 23,4 - 34,5 % females (table 3). The amount of steriles ranged from 16,4 - 23,4 %. Statistical analysis did not show significant deviation from 50 : 50 sex ratios. However, the ratio male : female : sterile did not deviate significantly from 50 : 25 : 25 either. Crossing an E4-*X/Y*-6 male with female E4 (E4-*X/Y*-7) yielded equal amounts of males and females.

The true YY-nature of a male from an androgenetic E4-*X/Y*-3 offspring was revealed after crossing this male to three females from different genetic backgrounds (table 4). Apart from some sterile animals, all-male populations were obtained. Androgenetic reproduction of this male yielded an all-male clone.

Table 3: Numbers of animals analysed (n) and percentages of male, female, intersex and sterile individuals after androgenetic reproduction of 3 males from the E4-*X/Y*-6 offspring and after crossing a E4-*X/Y*-6 male to an E4 female to produce E4-*X/Y*-7. All sex ratios were tested against the expected 50 : 50 sex ratio (XX : XY in E4-*X/Y*-7; XX : YY in androgenetic offspring) using Yates' corrected χ^2. Sterile individuals were left out of the analysis.

Cross	n	%-males	%-females	%-intersex	%-steriles	χ^2	P
E4-*X/Y*-6-1	47	53,2	23,4	0	23,4	2,08	0,15
E4-*X/Y*-6-2	55	49,1	34,5	0	16,4	0,40	0,53
E4-*X/Y*-6-3	51	49,0	33,3	0	17,6	0,43	0,51
E4-*X/Y*-7	48	35,4	50,0	2,1	12,5	0,31	0,58

Table 4: Numbers of animals analysed (n) and percentages of male, female and sterile individuals after crossing a putative YY-male from an androgenetic E4-*X/Y*-3 offspring with females from 3 different strains (E4, A and K31) and after androgenetic reproduction of the putative YY-male.

Cross	n	%-males	%-females	%-steriles
YY×E4	60	98,3	0	1,7
YY×A	298	99,0	0	1,0
YY×K31	246	95,7	0	4,1
YY-andro	25	100,0	0	0

Discussion

During inbreeding, sex ratios in the congenic strains remained stable (50 : 50, table 1). It can be calculated that animals in the E4-*X/Y*-6 strain are nearly 99 % identical to the homozygous female E4. Gynogenetic reproduction of this female has yielded repeatedly all-female clones, indicating a stable female genotype of E4 (Komen et al., 1992; Komen and Bongers, unpublished results). From this, it can be concluded that maleness in the congenic strain is induced by major (Y-derived ?) gene effects.

Viable homozygous YY-genotypes of common carp were produced by androgenetic reproduction. When crossing an androgenetic male to females from different genetic backgrounds, all-male populations were obtained. Few animals developed sterile gonads (table 4). Parsons and Thorgaard (1985) proved homozygous YY-individuals to be viable by karyological analysis of developing androgenetic *O. mykiss* embryos. Progeny of some androgenetic males were shown to be all-male, revealing their YY-nature. However, others yielded only females (Scheerer et al., 1991). The authors concluded that autosomal genes or environmental influences might have caused androgenetic XX animals to develop into males.

High numbers of sterile individuals were found in all androgenetic offsprings, especially when E4-*X/Y*-3 males were used as sperm donor (table 2). When E4-*X/Y*-6 males were used, sex ratios did not differ significantly from a 50 : 50 sex ratio (table 3). However, in all E4-*X/Y*-6 androgenetic offsprings, males were in excess of females and sex ratios did not differ significantly from 50 : 25 : 25 either. This could indicate that androgenetic YY-genotypes give rise to male gonadal development, and XX-genotypes to females or steriles. Reduced fertility is often observed as a result of inbreeding (Falconer, 1986). However, the E4-*X/Y*-3 and E4-*X/Y*-6 strains, which are both highly inbred, were nearly free of steriles. Evolution of sex chromosomes is associated with (1) decreasing homology between the sex chromosomes resulting in (partly) absence of genetic exchange, (2) dosage compensation of the activity of the X-chromosome and (3) the accumulation of highly repeated DNA-sequences without functional significance on the Y chromosome (Charlesworth, 1991). In mammals, evolution of sex chromosomes has reached a stage where the Y-chromosome contains only very few genes (SRY, H-Y: Goodfellow and Lovell-Badge, 1993). For this reason, mammalian YY-genotypes are not viable. In fish, only in few species morphological distinct sex chromosomes have been identified (Yamazaki, 1983; Chourrout, 1988). Repetitive DNA-sequences, associated with sex chromosomes have only been found in *Xiphophorus* sp. (Schartl, 1988), *Oncorhynchus tschawytscha* (Devlin et al., 1991), *Poecilia* sp. (Nanda et al., 1990; 1993) and in *Leporinus elongatus* (Nakayima et al., 1994). YY-individuals are viable in many fish species. These facts combined suggest that sex chromosomes

in fish are still evolving. It is known that in cases of genetic sex determination where dioecy has evolved recently, proto-X chromosomes carry female fertility and male sterility genes and proto-Y chromosomes carry male fertility and female sterility genes (Charlesworth, 1991). Chromosomal rearrangement during meiosis could then give rise to sperm cells, carrying X-chromosomes with female sterility genes. This could lead to sterile XX individuals after androgenetic reproduction, assuming that Y-chromosomes carrying X-derived male sterility genes still develop into males due to the major effect of male fertility genes. This could explain why in E4-*X/Y*-6 androgenetic reproductions consistently less females than males were found, the difference being sterile individuals. However, it does not explain the high numbers of sterile individuals in the E4-*X/Y*-3 androgenetic and control crosses. In these crosses, eggs from E4-*X/Y*-1 females were used. We have observed high numbers of sterile individuals in other crosses where these females were used as broodstock (J. Komen, unpublished results), so a cytogenetic cause cannot be excluded. Irradiating eggs for androgenetic reproduction could increase the amount of sterile animals by damaging cytogenetic factors (Egami and Ijiri, 1979; Bongers et al., 1995).

Our results give additional proof for a XX/XY sex determining mechanism in common carp. Although in other species viable YY male genotypes have been produced, no persistent YY strains have become available. In our laboratory, we now use androgenesis to establish such strains in our attempt to standardize strains of common carp.

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Chapter 5

Maternal influence on development of androgenetic clones of common carp, *Cyprinus carpio* L.

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Abstract
To test for a residual maternal influence in the performance of androgenetic offspring in common carp, *Cyprinus carpio*, an experiment was conducted where three females and three isogenic males were used to produce nine androgenetic families. Eggs were irradiated with UV (175 mJ/cm^2) and diploidy was induced using a heat shock (40 °C, 2 min). Yields of androgenetic diploids depended mainly on the egg donor. After raising the larvae for a period of 10 weeks, significant differences were observed regarding the frequency of normal and deformed animals, also depending on the egg donor. It is hypothesized that the UV-irradiation causes damage in maternal RNA's, resulting in disturbed embryonic development.

1. Introduction

Isogenic strains of fish can be produced by crossing homozygous individuals, obtained by gynogenesis and androgenesis. In our laboratory, gynogenesis was optimized by Komen et al. (1988, 1991) and recently we succeeded in optimizing androgenesis using UV-irradiation and heat shocks (Bongers et al., 1994). Androgenesis, using a homozygous (androgenetic) sperm donor results in the production of clones, thus facilitating the conservation of specific genotypes. In androgenesis, eggs are irradiated to destroy the female nuclear material. During oogenesis a large stock of maternal RNA (mainly rRNA), proteins (i.e. enzymes) and mitochondrial DNA is stored in the cytoplasm and plays an important role during early development (Davidson, 1986). Stroband et al. (1992) showed that maternal products only control development in common carp zygotes untill the stage of epiboly, which occurs 5-6 h after fertilization at 25 °C. During UV-irradiation damage is done to these maternal products as shown for example in *Oryzias latipes* (Egami and Ijiri, 1979). Fertilized common carp eggs are, during the early stages of development, far more sensitive to γ-rays than during later stages, the one-cell stage being the most critical (Frank, 1971).

For genome manipulation purposes it is thought that there is a dose-range where the nuclei can be genetically inactivated without damaging the cytoplasm to a significant extent. However, we observed differences in viability and performance of androgenetic clones, using the same homozygous sperm donor but different egg donors (unpublished results). This could be the result of differences between egg batches in UV-susceptibility of cytoplasmic maternal products.

To quantify this maternal influence, an experiment was designed where eggs of three females were irradiated and fertilized with sperm of three genetically identical males. Performance of the resulting nine androgenetic offsprings was compared using growth parameters and frequency of malformations.

2. Materials and methods

2.1. Broodstock

Males (age: 10 months) originated from a homozygous clone named E6. They were produced by androgenetically propagating a homozygous gynogenetic male. Gynogenetic males are thought to be homozygous for a recessive mutation (mas-1) affecting sex differentiation: XX;mas-1/mas-1 males (Komen et al., 1992). From this E6-clone, 3 males were used as sperm donor. Isogenicity of these males was confirmed using skin grafting, performed as described by Komen et al. (1990). Each male received one allograft from both siblings and one autograft as control for the grafting technique.

Females (age: 15 months) originated from three heterozygous clones (E20E6, E4E5 and E20E5), obtained by crossing homozygous gynogenetic males (E5 or E6) with homozygous gynogenetic females (E4 or E20) (Komen et al., 1991). They are thought to be heterozygous for the masculinization-inducing mutation: XX;mas-1/mas-+ females.

Males were homozygous for a recessive mutation affecting coloration and therefore have a blond appearance. Females used were heterozygous for this mutation and were thus black. Absence of black fry in UV-irradiated groups thus confirms elimination of the female genome (Bongers et al., 1994).

All broodstock were maintained at 25 °C in 140 liter aquaria with recirculating water and were fed carp pellets (25% crude protein, Provimi b.v., Rotterdam, the Netherlands) 0.9% of their body weight per day.

2.2. Androgenesis experiment

Eggs and sperm were obtained as described by Komen et al. (1991). After stripping, eggs were kept at room temperature. Milt was diluted 1:3 with physiological saline (0.9% NaCl) and stored on ice.

Irradiation was done as previously described (Bongers et al., 1994). Briefly, egg samples were put in a petridish and 5 ml of a synthetic ovarian fluid (OF) was added. The petridish was then placed on a mechanical stirrer and eggs were irradiated while gently stirring, facilitating the eggs to roll over in the OF. The intensity was kept constant at 1.1 mW/cm^2. After irradiation, egg samples were immediately mixed with 0.25 ml of sperm suspension and fertilized by adding water (24.0 °C). They were transferred to baskets (diameter 10 cm) with a mesh-bottom (mesh-size 0.5 mm) and incubated at 24.0 °C in the dark.

Nine androgenetic offsprings were produced by fertilizing irradiated egg samples from the three females with sperm of the three isogenic males. The total UV-dose administered was 175

mJ/cm^2, reflecting an irradiation-duration of 2 min 40 sec. Heat shocks were administered by transferring the eggs from the incubation-system to a waterbath of 40.0 °C for 2 minutes.
For all nine combinations of parents, the following groups were produced:

1. Androgenetic groups. Approximately 500 eggs were irradiated, fertilized and heat shocked at 28, 30, 32 or 34 min after fertilization.
2. Normal control group. To check the quality of eggs, yields of normal fry from non-irradiated eggs fertilized with milt were recorded.
3. Mechanical stirring control. To determine the effect of stirring on hatchability, eggs were mechanically stirred in OF for 2 min 40 sec, without irradiation. A negative effect of only adding OF (no stirring) is already known to be absent (Bongers et al., 1994).
4. UV-control: The efficiency of the UV-irradiation was determined by incubating non-shocked irradiated eggs, fertilized with milt.

Only the normal control groups were performed in duplicate.

2.3. Raising of larvae

During the first 4 weeks after hatching, diploid androgenetic and control larvae were raised in 25 l aquaria at 25.0 °C. Androgenetic larvae were pooled per parental combination. Control larvae were obtained from the normal control and the mechanical stirring control. They were fed freshly hatched *Artemia* nauplii *ad libitum*. After this four week period, larvae were moved to another recirculation system with 70 l. aquaria (T: 25.0 °C). For a total period of 6 weeks, they were fed vitamin C-enriched trout pellets (Trouvit, Trouw and co., Putten, the Netherlands) *ad libitum*. The flow rate was 5 l/min, keeping the O_2-concentration above 5 p.p.m. During this 6-week period, densities in the control groups were constant at 25 fish per aquarium. In androgenetic groups, densities depended on the number of surviving larvae.
From this period (10 weeks after hatching) until the age of 20 weeks, they were fed 25 g of trout pellets per $kg^{0.8}$ per day. After this period, food was reduced to 12.5 g per $kg^{0.8}$ per day to reduce the growth rate of the animals.

2.4. Parameters recorded and statistical analysis.

At 24 hours after fertilization, survival of developing eggs was recorded in the androgenesis experiment. At 96 hours after fertilization, the percentage of haploid androgenetic larvae, diploid androgenetic larvae, diploid biparental larvae and non-developed eggs were recorded. All percentages were expressed as percentage of the total number of incubated (treated) eggs.
At 4 weeks of age, androgenetic larvae were counted to calculate survival percentage. Mean body weight was determined per aquarium. From the control larvae, a sample was taken for

determination of mean body weight.

At 10 weeks of age, all animals were individually weighted and measured. Due to an unexpected high influence of initial density on growth, these data were not further used in testing for a residual maternal influence. Coefficients of variation (cv = (standard deviation / mean)×100) were determined. Frequencies of normal and deformed animals were recorded. Deformations consisted of deformed heads, compressed fins, reduction of gill covers etc. (Kirpichnikov, 1981). Frequencies were compared using Pearson's Chi-square test, using BMDP computer programs (Dixon et al., 1988). P-values < 0.05 are regarded significant.

3. Results

3.1. Androgenesis experiment

Egg qualities were high: hatching percentages of normal larvae in the normal controls ranged from 44.2 to 94.2% (mean: 67.7 ± 12.5%). The mechanical stirring control yielded 82.4 to 96.2% (mean: 91.4 ± 4.5%) normal larvae. In the UV-control, hatching of androgenetic haploids ranged from 57.2 to 92.2% (mean: 74.8 ± 10.8%) while few normal black larvae hatched (0.0 - 1.5%; mean 0.3 ± 0.5%) indicating elimination of the female genome.

Figure 1 depicts the survival and hatching percentages of the androgenetic groups. It can be read that early shocks (especially at 28 minutes after fertilization) result in high survival percentages after 24 hours but low hatching percentages of androgenetic diploids. In these groups, hatching of androgenetic haploids was high (up to 35.8%, data not shown). The highest hatching percentages of androgenetic diploids were obtained using eggs from female 3. Groups produced using eggs from female 1 had too low hatching percentages to be raised and were excluded from the experiment. No black (biparental) larvae hatched in any of the androgenetic groups.

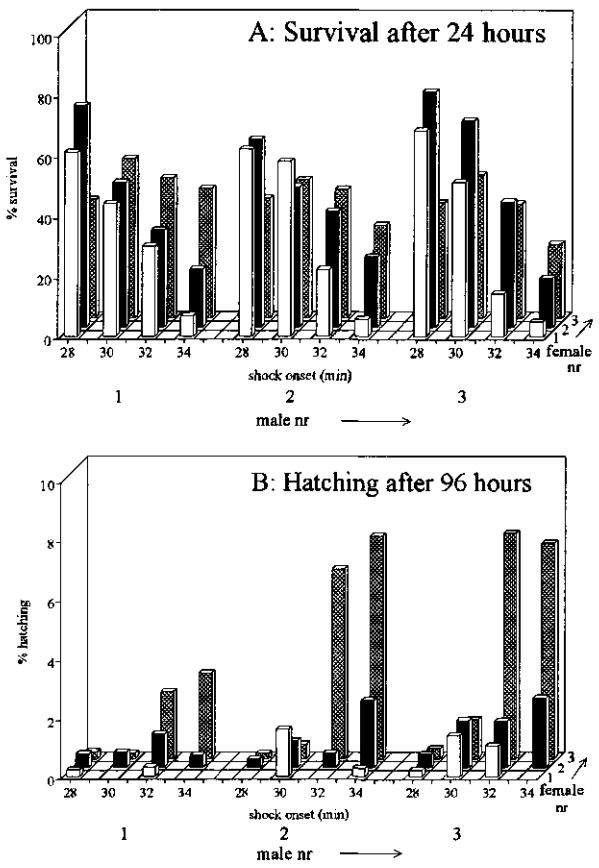

Figure 1: Survival-% after 24 hours (A) and hatching-% after 96 hours (B) of androgenetic larvae. Eggs were irradiated (175 mJ/cm^2) and heat shocked (40 °C, 2 min) at 28, 30, 32 or 34 minutes after fertilization.

3.2. Raising of larvae

Table 1 summarizes the performance of androgenetic and control larvae. Survival of androgenetic diploids was high, ranging from 75 - 100% during the first 10 weeks and from 75 - 96.6% during the first 10 weeks.

Weights of the androgenetic larvae after the first four weeks ranged from 1.8 to 4.1 grams, depending on the density in the aquarium. At 4 weeks, androgenetic larvae from $\sigma^*_2 \times \varphi_3$ and from $\sigma^*_3 \times \varphi_3$ were divided over two 70 l aquaria to approximate equal densities over all groups. Still, at 10 weeks of age body weights of androgenetic offspring varied considerably (19.6 - 31.0 g), according to the initial densities.

Growth in the control groups was more homogeneous: weights ranged from 0.5 to 0.8 g at 4 weeks and from 15.0 to 19.4 g at 10 weeks of age.

The coefficient of variation (cv) for individual body weight at 10 weeks of age was much lower in the control groups (15.3 - 24.7%) than in the androgenetic groups (30.9 - 64.4%).

Table 1: Survival and body weight (BW) of androgenetic and control larvae after the first period of 4 weeks and the subsequent period of 6 weeks (week 10).

	week 4				week 10				
group	initial nr.	end nr.	%-surv.	BW (g)	end nr.	%-surv	BW (g)	cv	range BW
Androgenetic									
$\sigma^*_1 \times \varphi_2$	14	12	85.7	4.1	12	85.7	31.3	64.4	1.5-60.5
$\sigma^*_2 \times \varphi_2$	21	18	85.7	2.7	16	76.2	26.4	44.8	3.3-50.3
$\sigma^*_3 \times \varphi_2$	24	18	75.0	3.5	18	75.0	31.0	41.6	5.3-45.8
$\sigma^*_1 \times \varphi_3$	26	26	100	2.6	23	88.5	24.2	38.5	6.0-41.5
$\sigma^*_2 \times \varphi_3$	58	57	98.3	1.8	56	96.6	19.6	30.9	6.5-32.9
$\sigma^*_3 \times \varphi_3$	53	51	96.2	2.0	48	90.6	22.5	41.5	3.4-40.6
Control									
$\sigma^*_1 \times \varphi_2$	na	25	na	0.57	24	96.0	15.1	24.7	7.8-25.9
$\sigma^*_2 \times \varphi_2$	na	25	na	0.65	25	100	17.2	19.3	11.0-23.6
$\sigma^*_3 \times \varphi_2$	na	25	na	0.73	25	100	17.6	18.9	10.2-23.3
$\sigma^*_1 \times \varphi_3$	na	25	na	0.77	25	100	19.4	15.3	13.9-24.8
$\sigma^*_2 \times \varphi_3$	na	25	na	0.52	25	100	15.2	20.8	8.7-22.1
$\sigma^*_3 \times \varphi_3$	na	25	na	0.71	25	100	17.7	23.2	12.1-25.1

Initial nr., number of individuals hatched; end nr., number of individuals at the end of the respective period; cv, coefficient of variation; na, data not available

Numbers of normal and deformed androgenetic offspring at 10 weeks are presented in table 2. In the control groups, only one deformed individual was observed in the cross $\sigma_3 \times \varphi_2$. Eggs from female 3 yielded signicantly more deformed androgenetic offspring than eggs from female 2 ($P<0.05$), indicating a residual maternal influence. Any male influence was absent. In the majority of the cases (67.6%), deformed fish were smaller than the average. Coefficients of variation for length and weight were higher in the androgenetic groups (means resp. 17.9 and 43.6 %) than in the control groups (resp. 7.0 and 20.4 %). Recalculation of the cv for length and weight, only using fish with a normal appearance revealed that cv's were still higher in the androgenetic groups than in the control groups (resp. 11.8 and 33.5 % vs 6.7 and 19.9 %).

Table 2: Numbers of normal and deformed larvae in the androgenetic groups, 10 weeks after hatching. Observed numbers are grouped by female and by male.

			grouped by female			grouped by male		
	normal larvae	deformed larvae		normal larvae	deformed larvae		normal larvae	deformed larvae
$\sigma_1 \times \varphi_2$	11	1				σ_1	28	7
$\sigma_2 \times \varphi_2$	14	2	φ_2	41	5			
$\sigma_3 \times \varphi_2$	16	2				σ_2	58	14
$\sigma_1 \times \varphi_3$	17	6						
$\sigma_2 \times \varphi_3$	44	12	φ_3 [a]	95	32	σ_3	50	16
$\sigma_3 \times \varphi_3$	34	14						

[a] Denotes significantly different from concomittant group ($P < 0.05$, Pearson's Chi-square).

4. Discussion

In the present research, we found differences in hatching of genetically identical homozygous androgenetic clones. The female egg donor significantly determined the frequency of deformations in androgenetic offspring, 10 weeks after hatching. These observations indicate the existence of a residual maternal influence.

Deformities are frequently observed after irradiating embryos. Allen and Mulkay (1960) irradiated embryos of the paradise fish *Macropodus opercularis* at different stages of development using a dose of 1 kR of X-rays. When applied directly after fertilization, anomalous growth of eyes, deformed central nervous systems and muscular deformaties were noted. Similar observations were reported by Frank (1971) after γ-irradiating common carp eggs and by Egami

and Ijiri (1979) after UV-irradiating eggs of *O. latipes*.

UV-irradiation causes damage to nucleotides. Several types of damage, including pyrimidine-dimers, DNA-DNA cross-links, pyrimidine adducts etc. have been observed in a wide variety of species. However, pyrimidine-dimer formation (T-T, C-T, C-C) in adjacent DNA-bases is the most common type of UV-damage (Friedberg, 1985). Similar damage occurs in RNA regarding the pyrimidines uracil and cytosin. Damage to mitochondrial DNA after UV-irradiating eggs of *O. niloticus* was shown to be absent, probably because of protection by the mitochondrial membrane (Myers et al., 1995). In teleosts, the pyrimidine-dimer formation is repaired by the enzyme DNA-photolyase under the influence of visible light (specifically at 300-600 nm). This enzyme also repairs damage in RNA (Friedberg, 1985). In our experiment, we incubated the eggs in the dark to prevent photoreactivation of the female nuclear DNA, thereby also preventing the repair of RNA. Damaged maternal RNA's lack the ability to produce essential proteins, necessary for proper development. As during the first 5-6 hours of development, common carp zygotes depend on maternal RNA's (Stroband et al., 1992) this could eventually result in deformed animals (Quah et al., 1992). In a number of invertebrates and lower vertebrates, development is of the determinative type, where ooplasmic determinants segregate among different blastomeres during early cleavage (see f.e. Jeffery, 1985). In *Xenopus laevis*, UV-irradiation causes a decrease in the number of germ cells (Ijiri, 1976). A dose of 41.1 mJ/cm^2, administered 0-10 min after fertilization destroyed the germ cell determinant, thereby inducing complete sterility. However, in teleosts development is considered to be of the regulative type, where cell determination and differentiation occur predominantly according to the cell position within the embryo. Cell interactions play an important role in these processes (Gevers, 1992). In conclusion, it can be hypothesized that RNA-damage has influenced early development (gastrulation, involution), by affecting the fate of individual cells or cell lineages and thus differentiation processes. RNA in eggs of female 3 would then have been more susceptible to UV-irradiation than that in eggs from female 2, leading to a higher incidence of deformed androgenetic offspring. However, eggs of female 3 yielded more androgenetic diploids at hatching. A positive correlation between quality and quantity of the offspring thus seems to be absent. Possible differences in abundance of maternal RNA's involved in different early developmental processes could have been responsible for this. Nevertheless, others factors influencing development cannot be excluded.

In the present experiment, high hatching percentages of androgenetic haploids at the early shock onsets confirms our previous results of low susceptibility of eggs to heat shocks during prometaphase (Bongers et al., 1994).

Reducing the total UV-dose does not seem appropriate in attempting to decrease the frequency of deformations as (1) maternal nuclear DNA-fragments might then contribute to the zygote

(Disney et al., 1987) and (2) in single-stranded DNA and RNA, pyrimidine-dimer formation reaches an equilibrium at relatively low UV-dosages (Friedberg, 1985).

A high correlation between initial density and body weight at 4 and 10 weeks after hatching was observed. A similar correlation was found in *Clarias gariepinus* by Haylor (1991), although weight differences were not as pronounced as in this study. Haylor (1991) also found a slight negative correlation between stocking density and the coefficient of variation for length and weight. In our study, cv for length and weight doubled in the androgenetic groups, compared to the control groups.

Isogenic strains are important tools to standardize experimental research (Festing, 1979). From our results it can be concluded that homozygous (androgenetic) isogenic strains are not suitable for this purpose since coefficients of variation are consistently much higher than in the control groups. This could be due to damage done by the UV-treatment (Egami and Ijiri, 1979) and the heat shock treatment (Komen et al., 1993). Another explanation for increased morphological variation in homozygous animals is reduced homeostasis. Due to the small variation in alleles (i.e., enzyme variants), homozygous animals are less equipped to adapt to small environmental fluctuations (Lerner, 1954). Therefore for researching purposes, F1-hybrids should be produced from 2 homozygous parents to create heterozygous isogenic strains. A similar experiment, using homozygous egg donors would have facilitated a comparison between heterozygous and homozygous isogenic strains. However, homozygous egg donors repeatedly failed to produce androgenetic offsprings, due to inferior egg quality (Bongers et al., 1994).

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Chapter 6

Origin of variation in isogenic, gynogenetic and androgenetic strains of common carp, *Cyprinus carpio*.

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Abstract

After androgenetic or gynogenetic reproduction, a large expansion of phenotypic variance is generally observed. Within one homozygous family, this expansion is the result of increased environmental variance since genetic variance does not increase. We consider three types of environmental variance (V_E) within homozygous offsprings: (1) "true" V_E (inter-individual variance), (2) V_E, due to developmental instability (DI, intra-individual variance) and (3) V_E originating from embryonic damage (ED) caused by the chromosome manipulation treatment. We examined the importance of these 3 types of V_E. It is thought that homozygous individuals show high levels of true V_E and DI. Therefore, in a first experiment we compared three F1-isogenic and one partly outbred strain of common carp, *Cyprinus carpio* in true V_E of length, body weight and number of dorsal fin rays. The isogenic strains varied in degree of homozygosity (coefficient of inbreeding F: 0 to 0.99). DI was determined by measuring fluctuating asymmetry (FA) of 5 bilateral symmetric characteristics. We found the strain with the highest F to display the lowest true V_E. FA was equal in all isogenic strains but highest in the partly outbred strain. In a second experiment, similar observations were performed on gynogenetic and androgenetic offsprings from parents with identical genotypes. Homozygous (endomitosis, EM: F = 1) and partly heterozygous (2pb-gynogenesis: F = 0.79) gynogenetic groups were produced. Normal fertilizations (F = 0.75) served as controls. The androgenetic groups showed highest FA and variations caused by ED, followed by 2pb- and EM-gynogenetic groups respectively. We conclude that increased variation within gynogenetic or androgenetic offsprings is the result of ED, caused by the chromosome manipulation treatment.

Introduction

In fish and amphibia, gynogenesis and androgenesis are efficient methods to produce inbred animals. In gynogenesis, eggs are fertilized with irradiated (genetically inactive) sperm. In androgenesis, the female genome is inactivated. Using physical shock treatments, diploidy can be restored by retention of the second polar body (2pb-gynogenesis) or by suppression of the first mitotic division (EM-gynogenesis and androgenesis). Since with this last method a doubled haploid genome remains undivided over two daughter cells (EndoMitosis), fully homozygous embryos are produced. Homozygous animals can serve as broodstock for the production of standardized strains. Genetic standardization of strains, as for example in mice and rats is still undeveloped in poikilotherm laboratory animals.

In homozygous androgenetic or gynogenetic offsprings, a large expansion of phenotypic variance is generally observed (Taniguchi et al., '90; Komen et al., '92a; Quillet, '94; Hussain et al., '95 and Bongers et al., '96). In theory, phenotypic variance in homozygous offsprings only consists of additive genetic variance (V_A), environmental variance (V_E) and the interaction between these two components (V_{AE}). Recently, we showed that V_A within one homozygous family equals V_A in the original population, assuming that the parent was fully outbred (Bongers et al., '96). Hence, increased phenotypic variance only results from increased V_E and/or V_{AE} . Further, when homozygous parents are used in gynogenesis or androgenesis, the resulting homozygous clonal offsprings (no genetic variance !) still display high residual variation and large amounts of

deformed individuals (phenodeviants) (Komen et al., '93; Taniguchi et al., '94; Bongers et al., '95). Due to absence of V_A and V_{AE}, only V_E can be responsible for this.

We are interested in the origin and amount of this residual variation in clones of common carp, *Cyprimus carpio* since we aim to standardize carp strains. Three types of V_E have to be considered. Firstly, homozygous populations are in general more susceptible to V_E (Falconer, '89). This source of V_E will be referred to as "true V_E" and consists of inter-individual variation. Secondly, through reduced homeostasis of homozygous genotypes, the buffering of developmental processes against environmental and physiological sources of variability decreases, resulting in developmental instability (DI: Lerner, '54; Mitton, '93; Komen et al., '93; Hussain, '95). In general DI, or intra-individual variability, is measured by comparing bilateral symmetric characters within the same individual (fluctuating asymmetry, FA) and can be considered as an unbiased estimate of deformity (Leary et al., '85a). Lerner ('54) proposed that DI increases with increasing levels of homozygosity. At present, there are several examples in literature where this correlation is confirmed but negative correlations have also been reported (for review, see Palmer and Strobeck, '86). Thirdly, V_E can have been generated from negative side effects of the chromosome manipulation treatment on cytoplasmic components (Komen et al., '93; Mair, '93). This last source of V_E will be referred to as "embryonic damage", ED. In a previous study, we already found a residual maternal influence on the performance of androgenetic clones. It was hypothesized that UV irradiation of the eggs could induce additional variation in androgenetic offsprings (Bongers et al., '95).

In our laboratory we succeeded in the development of isogenic strains of common carp. Gynogenetic (Komen et al., '88, '91) and androgenetic (Bongers et al., '94) reproduction techniques have been optimized. Using these methods, all female and all male homozygous clones, as well as their F1-(isogenic) hybrids have been produced. These animals are excellent models to study the origin of increased variation in chromosome manipulated fish. In a previous paper, we examined the consequences of chromosome manipulation on genetic variation (Bongers et al., '96). In the present paper, we examine the three aforementioned types of V_E. In a first experiment, we compare three F1-isogenic and one partly outbred strain in variation of morphological characteristics and DI of several bilateral symmetric characteristics. The F1-isogenic strains varied in degree of homozygosity and could therefore yield information on the amount of true V_E and DI in relation to the degree of homozygosity. In the second experiment, gynogenetic and androgenetic offsprings were produced from parents with identical genotypes to determine the effect of the chromosome manipulation treatments on DI and ED.

Material and methods

Artificial reproductions and raising of fish

Normal fertilizations and gynogenesis were performed as described by Komen et al. ('88, '91); androgenesis as described by Bongers et al. ('94). In the genome manipulation treatments, sperm and eggs were irradiated with a total UV-dose of approx. 10,000 and 175 mJ/cm^2, respectively. Cold shocks (2pb-gynogenesis) were administered by transferring the eggs from the incubation-system (24 °C) to a waterbath of 0-2 °C at ½, 1, 1½, 7 or 9 minutes after fertilization, for a total duration of 45 minutes. Heat shocks (EM-gynogenesis and androgenesis) were administered by transferring the eggs to a waterbath of 40.0 °C for 2 minutes at 26, 28, 30, 32 or 34 minutes after fertilization. After the shock treatment, eggs were incubated in the dark to prevent photoreactivation of the DNA.

During the first four weeks after hatching, a maximum of 100 larvae per experimental group were raised in 25 l aquaria at 25.0 °C in a recirculation system. Larvae, produced at different shock onsets from the same treatment were pooled. They were fed freshly hatched *Artemia* nauplii *ad libitum*. After this period, they were moved to another recirculation system with 70 l aquaria (T: 25.0 °C) and were fed vitamin C-enriched trout pellets at a daily ration of 25 gr per kg$^{0.8}$. Food, daily adjusted according to the expected growth and observed mortality, was administered using Scharfflinger conveyer belt feeders, approx. 12 hours a day. All animals were counted and weighed biweekly. This feeding strategy is considered to result in optimal growth, according to the feed manufacturer (Trouw, Vervins, France).

Experimental design

In experiment 1, three F1-isogenic and one partly outbred strain with different levels of homozygosity (coefficient of inbreeding F) were produced to determine the relation between the level of homozygosity and the amount of true V_E and DI. Since only normal fertilizations were performed, ED was assumed to be absent. A female from the homozygous gynogenetic strain E4 served as egg donor. This clone originates from a pedigree where initially Dutch and German carp strains were crossed. Three homozygous males were crossed with this female to produce the F1-isogenic strains. The first male (R3R8) originated from a pedigree of Polish (R3) and Hungarian (R8) carp lines. It is therefore unrelated to female E4 and F of the resulting isogenic strain is 0. The second male (E5) was a homozygous full-sib of E4 and therefore F in the E4xE5 strain is ½ (Bongers et al., '96). The third male (E4.Y5) stems from a strain, congenic to E4. This strain was produced by repeatedly backcrossing male offspring to the homozygous clone E4. Male E4.Y5 stems from the fifth backcross, and it can be calculated that this male is 98 % identical to E4. Therefore, F in the E4xE4.Y5 strain approximates 1. In order to determine the

Table 1. Summary of experiment 1 and 2. Groups of fish were raised and at 14 weeks of age, morphological variation and fluctuating asymmetry (FA) were determined. F = coefficient of inbreeding. V_G = Genetic variation present (yes or no). V_E = type of environmental variation present: 1 = true V_E (inter-individual variation), 2 = V_E due to developmental instability (intra-individual variation), 3 = V_E due to embryonic damage from chromosome manipulation treatments. n = number of experimental groups. In experiment 1, only normal fertilizations were performed. Differences in variation are attributed to the degree of inbreeding. In experiment 2, comparing normal fertilizations with 2pb-gynogenetics yields the cold shock effect. Comparing EM-gynogenetics and androgenetics yields the effect of UV irradiating the eggs. Further explanation: see Materials and Methods.

	Experimental group:	♀	♂	F	V_G	V_E	n
Exp. 1	normal fertilizations	E4	R3R8	0	no	1,2	1
		E4	E5	0.5	no	1,2	1
		E4	E4.Y5	0.99	no	1,2	1
		E4	FS	0.375	yes	1,2	1
Exp. 2	normal fertilizations	E4E5	E4E5	0.75	yes	1,2	4
	2pb-gynogenesis	E4E5	-	0.79	yes	1,2,3	2
	EM-gynogenesis	E4E5	-	1.0	yes	1,2,3	2
	androgenesis	-	E4E5	1.0	yes	1,2,3	4

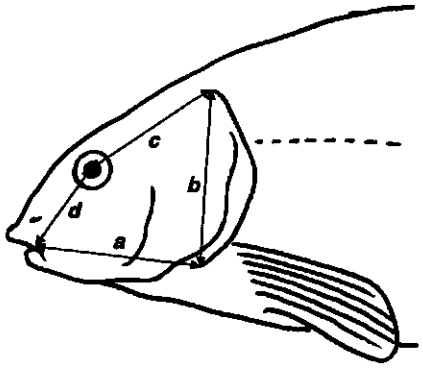

Figure 1. Graphic representation of the four metric fluctuating asymmetry indices measured in the experiments. A, B, C and D were measured at the left and right side, and fluctuating asymmetry was then calculated as $|L-R|/((L+R)/2)$.

influence of the introduction of genetic variance, female E4 was further crossed to a related, partly outbred male FS (F in E4xFS: 0.375).

In experiment 2, males and females from the isogenic E4xE5 strain were used as egg and sperm donors. This strain is normally all-female since E5 was characterized as being a gynogenetic XX-male (Komen et al., '92b). However, in previous experiments E4xE5 offspring was sex-reversed by administering a diet containing 17α-methyltestosterone (Komen et al., '93). As a result, males and females with identical genotypes were available. From two males and two females the following twelve experimental groups were produced: normal fertilizations with every possible combination of parents (n = 4), 2pb-gynogenetic (n = 2), EM-gynogenetic (n = 2) and androgenetic offspring with every possible combination of parents (n = 4).

Since F of E4xE5 equals ½, F in offspring from normal fertilizations (E4xE5 x E4xE5) equals 0.75. The F of 2pb-gynogenetic offspring depends on the recombination rate during meiosis I. From the work of Nace et al. ('70), assuming a mean distance of genes from the kinetochore and a mean coefficient of coincidence of chromosomes, F can be estimated as 0.79 (range: 0.72 - 0.85). Therefore, it can be assumed that the 2pb-gynogenetic offsprings are to a similar extent inbred as the offsprings from normal fertilizations. EM-gynogenetic and androgenetic offspring are fully homozygous (F = 1), but genetic variation is present since F of E4xE5 parents = ½. However, because parents are genetically identical, the amount of genetic variation in androgenetic and EM-gynogenetic offsprings is identical. Table 1 summarizes the experimental groups with their specific characteristics.

Parameters recorded and statistical analysis

At 14 weeks after hatching, body weight (0.1 g), length (mm) and number of dorsal fin rays were recorded for each fish. Means, variations and coefficients of variation (cv: (sd/mean)x100) were calculated. In experiment 1, variations were compared using Levene's F for variability. In experiment 2, cv's were grouped according to the treatment and differences were determined using ANOVA followed by Duncan's Multiple Range test.

Fluctuating asymmetry (FA) was determined for the number of pectoral fin rays (P) and for four metric indices A, B, C and D, measured at the left (L) and right (R) side of the head of every fish (0.1 mm) (Fig. 1). FA is one of three types of asymmetry, besides directional asymmetry (constant bias of greater development of the trait on side of the body) and antisymmetry (bimodal distribution of L-R differences). Prior to further analysis, distributions of L-R differences per trait, per experimental group were therefore tested for departure of normality. No significant skewness (directional asymmetry) or kurtosis (antisymmetry) was observed (Van Valen, '62; Palmer and Strobeck, '86).

FA per individual, per trait was then calculated as

$$(|L - R|/ ((L + R)/2)) \times 100\ \%$$

Correcting FA for size differences (i.e., expressing FA as percentage deviation from the mean of L and R) was performed as recommended by Palmer and Strobeck ('86) since size differences often distort detection of differences in FA among populations or strains.

In experiment 1, mean FA value per trait per experimental group was compared using a non-parametric t-test (Mann Whitney). In experiment 2, mean FA values per trait per experimental group were grouped according to the treatment and differences were determined using ANOVA followed by Duncan's Multiple Range test. Since the coefficient of inbreeding in the control crosses and the 2pb-gynogenetic groups are equal, observed differences in morphological variation and FA can be attributed to ED caused by the chromosome manipulation treatment. The EM-gynogenetic and androgenetic groups only differ regarding their mode of production (heat shock vs UV irradiation plus heat shock, respectively). Hence, differences are attributed to the damaging effect of UV (see also table 1).

All analyses were done using BMDP computer programs (Dixon et al., '88). P-values < 0.05 are regarded significant.

Results

Experiment 1

Lowest variation (true V_E) for length, body weight and the number of dorsal fin rays was observed in the homozygous isogenic strain (E4xE4.Y5, table 2). The influence of the introduction of genetic variation was demonstrated in strain E4xFS, which displayed the highest variations. Fluctuating asymmetry values were equal, except for index D where the highly inbred strains E4xE5 and E4xE4.Y5 showed the lowest levels of developmental instability (table 3).

Experiment 2

Coefficients of variation were higher in all chromosome manipulated groups compared to the control groups (table 4). There was a significant trend that variation in the androgenetic and 2pb-gynogenetic groups was highest. The number of dorsal fin rays was lower in chromosome manipulated groups, compared to the controls.

As for variation, FA increased in the manipulated groups (table 5). The control groups showed lowest developmental instability, followed by EM-gynogenetic, 2pb-gynogenetic and androgenetic groups respectively.

Table 2: Coefficient of inbreeding (F) and means plus coefficients of variation (cv) for length (L, mm), bodyweight (BW) and the number of dorsal fin rays (#D) in the four experimental groups of experiment 1. n = number of animals analyzed. Cv 's with a common superscript do not differ significantly (Levene's F, P<0.05).

	E4×R3R8		E4×E5		E4×E4.Y5		E4×FS	
F	0		0.5		0.99		0.375	
n	50		50		50		50	
	mean	cv	mean	cv	mean	cv	mean	cv
L	93.2	11.6	88.0	11.8	83.2	9.6	84.8	13.0
BW	35.0	29.4[b]	29.0	27.2	26.5	27.2	30.2	31.8
# D	21.0	5.2[abc]	21.1	11.4[c]	20.9	4.3[a]	21.3	6.6[bc]

Table 3: Fluctuating asymmetry values for the parameter P (= number of pectoral fin rays) and for the four metric indices A, B, C and D in the experimental groups of experiment 1. Groups with a common superscript do not differ significantly (Mann-Whitney t-test, P<0.05).

	E4×R3R8	E4×E5	E4×E4Y5	E4×FS
P	3.30	3.90	3.90	4.50
A	4.62	4.74	4.77	5.85
B	5.09	5.62	5.31	5.04
C	4.29	4.86	3.91	4.53
D	6.75[b]	4.54[a]	4.60[a]	6.53[b]

When comparing the control groups and the 2pb-gynogenetic groups, it can be deduced that a cold shock induces considerable ED. When assuming the effect of inbreeding on variation and FA to be absent, as in experiment 1, a heat shock also induces substantial ED. When comparing EM-gynogenetic and androgenetic groups, it can be concluded that UV irradiation causes additional damage.

Table 4: Mean length (L, mm), bodyweight (BW, g) and number of dorsal fin rays (#D) and the respective coefficients of variation (cv) in the 12 experimental groups of experiment 2. F = coefficient of inbreeding, n = number of animals analysed.
Controls = normal fertilizations, 2pb = 2pb-gynogenetic treatment, EM = EM-gynogenetic treatment, andro = androgenetic treatment. Treatments with a common superscript do not differ significantly (Duncan's multiple range test, $P < 0.05$).

	controls (F=0.75)				2pb (F=0.79)		EM (F=1.0)		andro (F=1.0)			
	♀1♂1	♀1♂2	♀2♂1	♀2♂2	♀-1	♀-2	♀-1	♀-2	♀1♂1	♀1♂2	♀2♂1	♀2♂2
n	60	65	59	58	55	47	60	58	19	31	12	30
Means:												
L	87.9	89.3	99.4	94.6	91.5	94.5	96.0	97.7	92.7	93.7	86.0	89.6
BW	25.4	26.4	36.9	31.7	36.2	45.3	39.5	41.9	39.0	43.1	32.8	33.0
#D	20.2	20.0	19.5	20.0 [(b)]	17.1	18.6 [(a)]	16.4	15.6 [(a)]	14.8	17.2	17.4	15.6 [(a)]
cv:												
L	9.7	12.1	13.1	10.0 [(a)]	22.0	31.4 [(bc)]	18.4	19.8 [(ab)]	31.5	29.7	32.7	21.2 [(c)]
BW	33.1	28.8	33.9	29.3 [(a)]	63.3	83.9 [(c)]	50.1	50.8 [(b)]	66.2	65.9	79.3	57.9 [(c)]
#D	12.4	16.5	15.9	13.5 [(a)]	33.9	44.5 [(b)]	20.4	44.9 [(ab)]	64.2	36.6	51.1	49.4 [(b)]

Table 5: Fluctuating asymmetry values for the parameter P (= number of pectoral fin rays) and for the four metric indices A, B, C and D in the 12 experimental groups of experiment 2. F = coefficient of inbreeding, n = number of animals analysed.
Controls = normal fertilizations, 2pb = 2pb-gynogenetic treatment, EM = EM-gynogenetic treatment, andro = androgenetic treatment. Treatments with a common superscript do not differ significantly (Duncan's multiple range test, $P < 0.05$).

	controls (F=0.75)				2pb (F=0.79)		EM (F=1.0)		andro (F=1.0)			
	♀1♂1	♀1♂2	♀2♂1	♀2♂2	♀-1	♀-2	♀-1	♀-2	♀1♂1	♀1♂2	♀2♂1	♀2♂2
n	60	65	59	58	55	47	60	58	19	31	12	30
P	4.7	3.5	4.3	3.6	5.8	6.2	5.0	4.6	6.7	3.7	7.1	6.4
A	3.3	4.8	3.5	3.3 [(a)]	5.7	7.2 [(ab)]	4.4	5.0 [(ab)]	5.1	7.6	10.1	6.4 [(b)]
B	5.1	4.7	4.3	3.4 [(a)]	7.3	7.6 [(b)]	5.7	7.4 [(ab)]	5.2	8.1	9.2	7.9 [(b)]
C	4.1	3.0	3.7	3.7 [(a)]	6.6	7.1 [(ab)]	4.2	4.7 [(ab)]	6.2	7.4	13.7	4.9 [(b)]
D	4.8	4.1	4.7	4.6 [(a)]	6.5	9.4 [(b)]	4.6	6.7 [(ab)]	7.3	6.9	10.2	6.9 [(b)]

Discussion

Festing ('76) compared homozygous inbred, F1 and F2 hybrids, and outbred strains of mice for variation in mandible shape, a polygenic quantitative trait. He found F1 hybrids to be the least variable, however not significantly less than homozygous inbred strains. They both were much less variable than F2 and outbred strains. Our results partly confirm these results on established laboratory strains. In experiment 1, the homozygous inbred strain E4xE4.Y5 showed to be the least variable for the traits length, weight and number of dorsal fin rays (table 2). FA indices were nearly equal for all strains (table 3). The outbred strain E4xFS was the most variable. Palmer and Strobeck ('86), who extensively studied literature on FA, concluded that FA tends to increase with increased inbreeding, although not all studies have yielded consistent results. Falconer ('89) stated that homozygous individuals are more susceptible to environmental sources of variation. From experiment 1, it can be concluded that the level of inbreeding does not seem to affect true environmental variation and developmental instability. The least variable homozygous isogenic strain E4xE4.Y5 is probably well adapted to our laboratory environment. The conclusions of Palmer and Strobeck ('86) and Falconer ('89) are probably only valid when inbreeding has not been completed, and inferior genotypes are still present in the population.

In experiment 2 it is clearly demonstrated that increased variation and DI in gynogenetic and androgenetic offsprings are the result of the chromosome manipulation treatment. It should be noted that in this experiment, broodstock (F1 hybrids) were free of recessive deleterious genes since their parents were homozygous. When comparing control and 2pb-gynogenetic groups it is clear that a cold shock gives rise to ED. A heat shock (EM-gynogenetic groups) seems to be less damaging than a cold shock. Comparing the androgenetic and the EM-gynogenetic groups yields the effect of UV irradiating the eggs, prior to fertilization. Since in androgenetic groups consistently more variation and FA was observed, it can be concluded that UV irradiation induces additional ED. Mair ('93) suggested this to occur after comparing hatching percentages of androgenetic and EM-gynogenetic *Oreochromis niloticus*.

Thusfar, our results are based on a limited number of genotypes. However, after analyzing 8 homozygous gynogenetic clones and 12 F1-hybrids for variation in gonadal development, we obtained supportive results for our present findings (Bongers et al., in prep.).

Survival after gynogenetic or androgenetic reproduction is low, in general not exceeding 5 - 10 %. The use of inbred parents (free of recessive deleterious genes) has not improved yields of androgenetic or gynogenetic diploids (Scheerer et al., '86; Komen et al., '93; Taniguchi et al., '94; Bongers et al., '95). Only from inbred zebrafish, *Brachydanio rerio*, yields were occasionally higher than from outbred individuals (Streisinger et al., '81). Therefore, it is likely that ED caused by the chromosome manipulation treatment accounts for most of the mortality.

Leary et al. ('85b) compared FA in normal diploid, gynogenetic diploid and triploid rainbow trout, *Oncorhynchus mykiss*. Gynogenetic diploids and triploids were produced by heat shocking eggs after fertilization with UV-inactivated and intact sperm, respectively. Gynogenetic diploids showed lower meristic counts and higher levels of FA than normal diploids. In contrast, triploids showed lower levels of FA than normal diploids. The authors stated that (1) deleterious effects of triploidy induction could not have been revealed by their methods of examining asymmetry or (2) that the increased level of heterozygosity in triploids as compared to diploids compensates for the increase in DI. However, gynogenetic diploids and triploids were not produced from the same egg batch, so direct comparisons should be done with precaution. Young et al. ('95) found that homozygous androgenetic clones of rainbow trout displayed higher variance and FA of meristic traits than heterozygous (F1-) isogenic strains. They attributed this to the increased susceptibility to V_E and increased DI of homozygous individuals. However, their experimental set-up did not allow detection of ED.

Temperature shocks are applied to destroy tubulin proteins. As a result, extrusion of the second polar body or the first cleavage of the zygote is inhibited (Makino and Ozima, '43). It is evident that other proteins (enzymes) will also be damaged by a temperature treatment. UV-irradiation causes damage to nucleotides, with pyrimidine-dimer formation (T-T, C-T, C-C) in adjacent DNA-bases being most common (Friedberg, '85). Similar damage occurs in RNA regarding the pyrimidines uracil and cytosine. Damaged maternal RNA's lack the ability to produce essential proteins, necessary for proper development. Since during the first 5-6 hours of development, common carp zygotes depend on maternal RNA's (Stroband et al., '92) this early ED could eventually result in increased variation and DI. Myers et al. ('95) showed that damage to mitochondrial DNA after UV-irradiating eggs of *O. niloticus* was absent.

We found lower meristic counts in the chromosome manipulated groups in experiment 2 (number of dorsal fin rays, table 4; number of pectoral fin rays, data not shown). This is in agreement with the findings of Leary et al. ('85b). They hypothesized that increased FA and lower meristic counts are related to decreased availability of energy during development. Reduced availability of energy could be the result of damage to enzymes or maternal RNA's, as a consequence from the chromosome manipulation treatment.

Soulé ('82) defined DI as developmental noise or error. He stated that it has no genetic or environmental origin and that it could account for most of the residual variation in inbred lines. Gärtner ('90) analyzed environmental variation for quantitative traits in laboratory strains of mice and rats and determined that only 20 - 30 % of the random variation was true environmental variation. The remaining 70 - 80 % was attributed to a component of variation, effective at or before fertilization and could originate from ooplasmic differences between eggs. The isogenic

strains used in experiment 1 display high residual variation. The variation for length and body weight in these 3 strains was only reduced with approximately 15 and 12 % respectively, compared to the partly inbred E4xFS strain. This confirms the findings of Soulé ('82) and Gärtner ('90). It can be hypothesized that differences in quality between eggs from the same batch account for most of the non-genetic variation. Chromosome-manipulation treatments increase differences in egg quality, thereby increasing the total variation in the offspring.

ED, resulting from chromosome manipulation treatments has important implications for the use of these techniques in selection programs, since animals are being produced with a phenotypic value not reflecting their genotypic value. Using females with high egg quality and/or strong selection (culling) during the growth phase reduces the possibility to draw incorrect conclusions from chromosome manipulated offsprings.

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Chapter 7

Distribution of genetic variance in gynogenetic or androgenetic families

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Abstract

Through gynogenetic and androgenetic reproduction, homozygous individuals are produced in one generation. Selection of homozygous gynogenetic or androgenetic individuals facilitates the production of genetically defined clones. However, to obtain a selection response a sufficient amount of additive genetic variance is needed. The additive genetic variance in homozygous populations doubles as compared to the additive genetic variance in the base population. We examined the possibility to use gynogenesis and androgenesis in estimating additive genetic variance. We derived additive genetic relations and the distribution of additive genetic variance between and within homozygous gynogenetic and androgenetic families.

Within gynogenetic families variance equals $V_A + V_E$ and between-family variation equals V_A. This theory is used to analyze experimental data on gonad development and fertility in homozygous gynogenetic common carp, *Cyprinus carpio*. Five gynogenetic families were produced from full sib, outbred female parents. Regular sampling for maturation stage between the age of 13 and 25 months revealed large between and within-family variance for the Gonado Somatic Index (GSI) and egg quality parameters. Heritabilities were estimated using Gibbs sampling. For the traits GSI at 13 months and the percentage of normal larvae, obtained after fertilization of egg samples at 19 months, heritability estimates were 0.71 and 0.72, respectively. This suggests that these traits are influenced by additive genetic effects and that selection can be used successfully to produce early or late maturing homozygous animals with high egg quality.

1. Introduction

Although fish are used in a wide array of scientific disciplines (Powers, 1989), genetic standardization of strains, as for instance in mice and rats is still undeveloped. In our laboratory, we are aiming to produce standardized inbred strains of common carp, *Cyprinus carpio* for research purposes. Common carp is an important experimental animal model in fundamental and applied research (Bongers et al., in prep.). Gynogenesis (Komen et al., 1991) and androgenesis (Bongers et al., 1994) have been optimized to produce homozygous individuals in one generation. Selection of homozygous individuals for specific traits facilitates the production of genetically defined clones (Wiegertjes et al., 1994). However, to obtain a selection response a sufficient amount of additive genetic variance (σ_A^2) is needed (Gjedrem, 1983; Falconer, 1989). The ratio $\sigma_A^2/\sigma_P^2 = h^2$ (heritability in the narrow sense) is a measure for the relative contribution of additive genetic variance to the total phenotypic variance. When the heritability is relatively high (usually > 0.5), selection of fish based on their own performance can be applied succesfully (Gjedrem, 1983). To maintain specific genotypes, selection of homozygous females with high egg quality is of critical importance. Quillet (1994) found a dramatic decrease in hatchability in eggs, obtained from homozygous rainbow trout, *Oncorhynchus mykiss* (19-36 % vs 66-72 % in controls). Komen et al. (1991) stated that less than 10 % of homozygous female carp can be gynogenetically propagated to produce clones, due to inferior egg quality resulting from inbreeding depression.

According to Falconer (1989), for a population consisting of fully inbred (isogenic) lines, additive genetic variance is doubled compared to the additive genetic variance in the base

population. Obviously, for such a population within-line genetic variance is zero, so all genetic variation is between-line variation. After homozygous gynogenetic reproduction all offsprings are fully inbred. However, due to segregation of maternal heterozygous alleles, within-line variation does not equal zero. Komen et al. (1992a) compared gonad development in homozygous gynogenetic and outbred common carp and found an increase in the phenotypic variation for several reproductive parameters in the gynogenetic offspring. Other authors reported increased variation for other traits in homozygous gynogenetic offspring when compared to outbred offspring (in carp, *Cyprinus carpio*: Sumantadinata et al., 1990; in ayu, *Plecoglossus altivelis*: Taniguchi et al., 1990; in rainbow trout, *Oncorhynchus mykiss*: Quillet, 1994; in *Oreochromis niloticus*: Hussain et al, 1995). This increased variation is likely due to an increased susceptibility of homozygous individuals to environmental sources of variation (Falconer, 1989), embryonic damage caused by the diploidy inducing treatment (Bongers et al., 1996) and to increased genetic variation resulting from inbreeding (Taniguchi et al., 1990, 1994; Quillet, 1994).

Quillet (1994) proposed that genetic variance within a homozygous gynogenetic family is half the genetic variance in the base population and, since total genetic variance doubles, between-family variation equals 1½ times the original genetic variance. Therefore, Quillet (1994) concluded that in order to estimate genetic variance, the creation of numerous homozygous gynogenetic families is relevant, even with few animals per family. However, no formal derivation of the distribution of genetic variance within and between gynogenetic families was given.

In this paper, we examine the possibility to use gynogenesis and androgenesis in estimating additive genetic variance. In the first part of the paper, additive genetic relations between and within gynogenetic (or androgenetic) families were derived. These relations are required to estimate heritabilities. Relations are deduced for homozygous gynogenetic offspring but also apply to androgenetic offspring. It will be shown that the partitioning of additive genetic variance between and within families is different from that proposed by Quillet (1994). In the second part of the paper, five homozygous gynogenetic families (see figure 1) were examined. These families were previously used to study genetics of antibody response (Wiegertjes et al., 1994). Our aim is to determine the possibility to select early and late maturing homozygous individuals with high egg quality for the production of homozygous clones with defined patterns of gonadal maturation. Therefore, the presented quantitative genetic framework was used to estimate

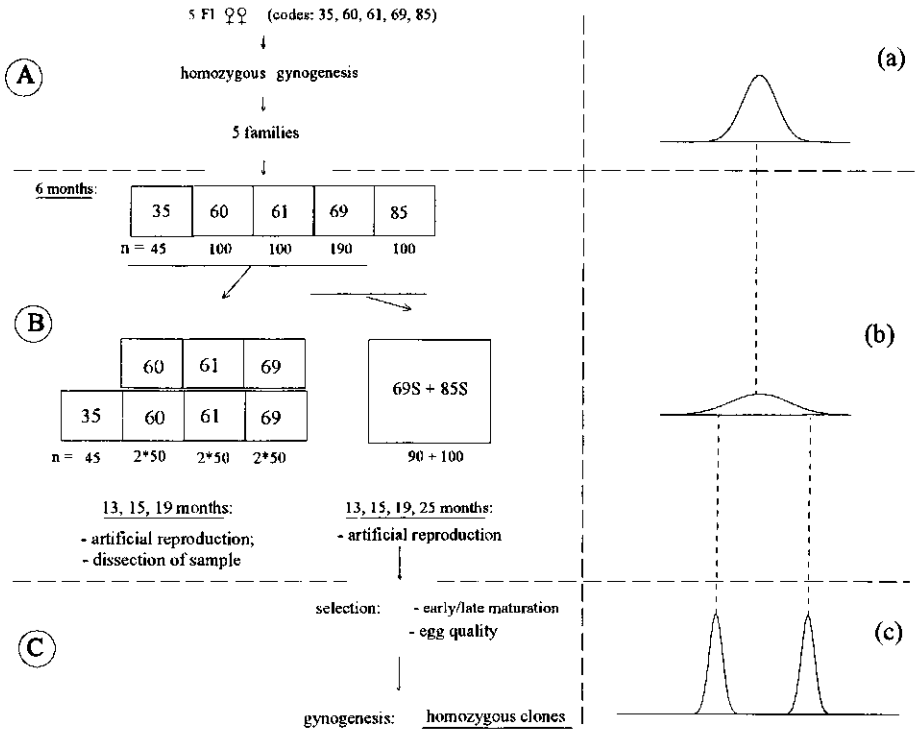

Figure 1: Schematic presentation of the experiment. For more detailed information: see section "Gonad development in homozygous families of common carp".

A: Five hybrid females were taken to produce homozygous families through gynogenetic reproduction.

B: After 6 months, the 5 families were subdivided as indicated. Maturation-stage was checked by artificial reproduction at 13, 15, 19 and (only 69S and 85S) 25 months of age. Families 35, 60, 61 and 69 were used to obtain more detailed information on maturation-stage by dissecting fish at each sampling.

C: Future research. Animals from 69S and 85S with high egg quality will be selected to produce clones with defined patterns of gonadal maturation.

Normal distributions in the right column show the theoretical phenotypic variation: variation in homozygous populations (b) is increased, compared to variation in F1 populations (a). When producing clones (c), variation is expected to be greatly reduced.

heritabilities using Gibbs sampling of the parameters gonad development and fertility in the homozygous families. These parameters were chosen since (1) in gynogenesis, selection of homozygous females with high egg quality is of critical importance and (2) age at maturation and fertility are important characters in fish breeding (Kirpichnikov, 1981). In common carp, late maturation is preferred since there is a negative correlation between growth rate and onset of sexual maturation (Hulata et al., 1985).

2. Theory

The coefficient of coancestry f_{xy} is defined as the chance that a random allele at locus A of individual X (i,k) is identical by descent to a random allele at locus A in individual Y (j,l):

$$f_{xy} = \frac{P(i=j) + P(i=l) + P(k=j) + P(k=l)}{4}$$

The additive genetic relation a_{xy} is defined as $2*(f_{xy})$ (Falconer, 1989). For example for full sibs from unrelated, non-inbred parents, $f_{xy} = ¼$ and $a_{xy} = ½$.

To illustrate genetic relations between gynogenetic individuals, a pedigree of the origin of gynogenetic families is depicted in figure 2. If a female parent has genotype (i,l) the gynogenetic offspring will be of type *(i,i) or (l,l)*, each with equal probability. The probability that a randomly drawn allele from female parent (i,l) is identical by descent to a randomly drawn allele from gynogenetic offspring (i,i) is equal to:

$$f_{parent\text{-}gynogenetic\ offspring} = \frac{P(i=i) + P(i=i) + P(l=i) + P(l=i)}{4} = \frac{2}{4}$$

The same holds true for the probability between the parent (i,l) and the other possible gynogenetic offspring (l,l). Because each type of offspring (i,i or l,l) has the same probability, the expected coefficient of coancestry between a parent and its gynogenetic offspring is ½. The additive genetic relationship between a gynogenetically produced individual and its parent thus equals 1. The additive genetic relationship between members of the same gynogenetic family can be derived by again assuming the parental genotype is (i,l). Gynogenetic offspring will be of type (i,i) or (l,l), each with equal probability. Four different genotype combinations for two individuals of the same gynogenetic family can then occur: (i,i) and (i,i), (i,i) and (l,l), (l,l) and (i,i), (l,l) and (l,l). Each of these combinations has the same probability of occurence and therefore the expected coefficient of coancestry between gynogenetic family members is (1 + 0

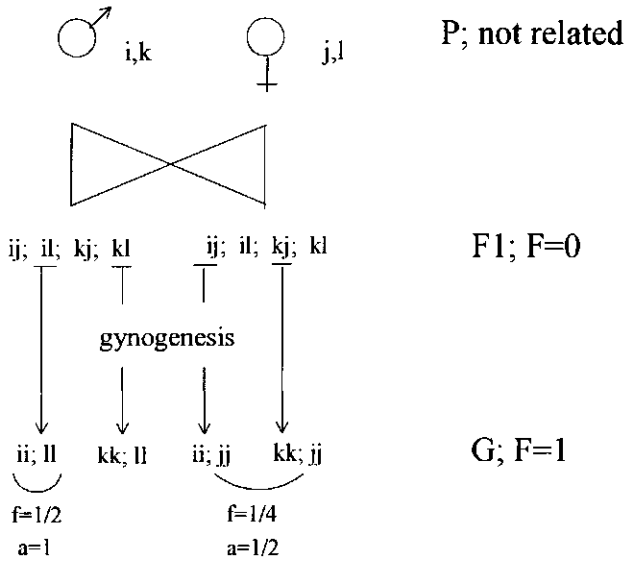

Figure 2. Determination of genetic relations in gynogenetic offspring. Parents (P) are assumed not to be related. F1-females are reproduced by homozygous gynogenesis to produce homozygous gynogenetic offspring (G). F = coefficient of inbreeding; f = coefficient of coancestry; a = additive genetic relation.

+ 0 + 1) / 4 = ½ and thus the additive genetic relation is 1. Similarly, it can be found that the coefficient of coancestry between gynogenetic families is ¼.

For the derivation of the distribution of genetic variance, it is assumed that the total variation in a gynogenetic population only consists of environmental variation (V_E) and additive genetic variation (V_A). Dominance variation (V_D) is thought to be absent and epistatic variation (V_I) negligible in homozygous populations (De Boer and Hoeschele, 1993).

In a base population containing numerous families (or lines), the additive genetic variance can be partitioned in between-family and within-family additive genetic variance. When slow inbreeding is practised, e.g. by full sib mating, the within-family additive genetic variance decreases and the between-family additive genetic variance increases:

$$V_{A\text{-Total}} = V_{A\text{-between families}} + V_{A\text{-within families}} =$$
$$2FV_A + (1\text{-}F)V_A = (1 + F)V_A \quad \text{(Falconer, 1989)}$$

In this situation, an inbreeding coefficient of 1 reflects a population consisting of several fully inbred (isogenic) lines. The within-line additive genetic variance is then zero and the between-line additive genetic variance twice the additive genetic variance in the base population. However, this formula does not apply when fast inbreeding is practised. Using gynogenesis, fully inbred individuals are obtained in one single generation and therefore gynogenesis can be considered as a typical example were this formula does not apply. Assume that a number of unrelated, non-inbred individuals are taken from a base population with additive genetic variance V_A. When these individuals are used in gynogenesis, families are created with fully homozygous (inbred) offspring. The between-family variance equals the variance of the family-means and the within-family variance the variance between homozygous individuals from the same parent. In this situation the following formula applies:

$$V_{A\text{-Total}} = V_{A\text{-between families}} + V_{A\text{-within families}} \approx$$
$$2fV_A + (1 + F - 2f)V_A \approx (1 + F)V_A$$

where f = the coefficient of coancestry among individuals of the same family (Falconer, 1989). Homozygous gynogenetic individuals are fully inbred (F = 1) and the coefficient of coancestry f_{xy} among individuals belonging to the same gynogenetic family is ½, as explained previously. As a result, the additive genetic variance within families is equal to the additive genetic variance between families and the total amount of additive genetic variance after gynogenetic reproduction is doubled.

The between-family variance can also be deduced from the following: the value of an individual, judged by the mean value of its progeny, is referred to as the breeding value. The variance of all breeding values in a population reflects the additive genetic variance (Falconer, 1989). When several (randomly drawn) females from a population are gynogenetically propagated, the variance of the progeny-means (= breeding values of the females), so the between-family variance, directly reflects the additive genetic variance.

3. Gonad development in homozygous gynogenetic families of common carp

Initially, a R_3 male (origin: Poland) was crossed with a not-related R_8 female (origin: Hungary). Five R_3R_8 females were then gynogenetically reproduced to produce homozygous gynogenetic families. Homozygous gynogenesis was performed as described by Komen et al. (1991). Per female, approx. 5000 eggs were fertilized with irradiated sperm and heat shocked. Males, heterozygous for the dominant trait scalation (Ss; origin: Wageningen-strain W) were used as

sperm donor. All females were mirror carps (ss). Absence of scaled individuals among gynogenetic offspring confirmed elimination of the male genome. Per family, 200 random animals were raised at 25 °C according to the standardized method used in our laboratory (Komen et al., 1992) (see also figure 1). At 6 months after hatching, deformed animals were discarded to exclude phenodeviants from the analysis. Phenodeviants are frequently observed during inbreeding, without showing a clear Mendelian mode of inheritance and can be recognized by deformations of the head, fins or the vertebral column. They are inferior as compared to normal fish in growth and viability (Kirpichnikov, 1981). We discarded phenodeviants mainly on the basis of retarded growth. From 6 months onwards, 100 animals from family 35, 60, 61 and 69 were randomly taken for further raising in two duplicate 140 l aquaria (50 fish per aquarium). All animals were sampled for maturation stage by artificial reproduction with carp Pituitary Suspension at 13, 15 and 19 months. Body weight (g) and weight of the stripped eggs (Weggs, g) were recorded and pseudo Gonado Somatic Index (pGSI: (Weggs / BW) * 100 %) was calculated. At each sampling, animals were sacrificed to determine Gonado Somatic Index (= (gonad weight / BW) * 100 %) and for histological examination of the gonads. Animals chosen to be sacrificed were all strippable (ovulated) animals supplemented with random animals. Family 69S and 85S were also sampled at 25 months of age. From these two families, only animals that never responded to cPS injections were sacrificed since these families were selected to serve as future broodstock on the basis of their immune responsiveness (Wiegertjes et al., 1994). They were grown together in an 800 l tank in the same recirculation system as the other families and were individually tagged using tattoos.

Table 1 shows data obtained in this experiment. Results of duplicate aquaria are combined since no significant differences were found between duplicates (t-tests, $P<0.05$). Differences in age at maturation between families were found. At 13 months, 16 % of the animals in family 35 could be stripped. Other families, especially family 61 could be stripped only at later samplings. At each sampling, strippable animals were consistently heavier than non-strippable animals and had higher GSI's. In the non-strippable (dissected) fish, variation in GSI between families as well as within families was high. Histological examination revealed that even at 19 months of age, there were animals with completely undeveloped gonads (% postvitellogenic eggs = 0). At all samplings, no individuals with ovaries in regression (high percentage of atretic oocytes) were observed.

Table 1: Results of sampling for maturation stage in homozygous gynogenetic families after injection with maturation inducing hormone (cPS). Results of duplicate aquaria are combined. N = initial number of females at the start of the sampling period. n (%) = number and percentage of ovulated females, BW = body weight, GSI = Gonado Somatic Index, pGSI = pseudo GSI, figures between brackets = coefficient of variation, %-post = % of postvitellogenic eggs, range-post = range from minimum to maximum % of postvitellogenic eggs. nr = not recorded.

			ovulated females				not ovulated females				
Age	Family	N	n (%)	BW	pGSI	GSI	n	BW	GSI	%-post	range-post
13 months:	35	31	5 (16)	472 (18)	2.7 (89)	6.7 (23)	5	312 (22)	5.9 (31)	12.0	8 - 24
	60	80	1 (1)	350	0.3	8.9	18	231 (22)	2.6 (89)	5.7	0 - 17
	61	61	0				3	289 (26)	1.4 (42)	0.5	0 - 1.5
	69	69	0				15	271 (36)	2.8 (82)	5.8	0 - 14.3
	69S	69	0				69	242 (49)	nr	nr	
	85S	95	0				95	257 (25)	nr	nr	
15 months:	35	20	8 (40)	615 (19)	4.1 (59)	11.8 (29)	5	285 (19)	2.9 (71)	1.2	0 - 5.8
	60	60	13 (22)	291 (20)	3.3 (82)	10.1 (20)	10	277 (30)	4.6 (71)	3.2	0 - 16.4
	61	37	0				5	359 (18)	1.4 (89)	0.7	0 - 2.7
	69	49	3 (6)	409 (27)	3.3 (24)	11.3 (4)	8	375 (79)	2.5 (84)	0.4	0 - 2.9
	69S	63	13 (19)	472 (41)	4.4 (70)	nr	50	336 (43)	nr	nr	
	85S	92	0				92	411 (24)	nr	nr	
19 months:	35	6	2 (33)	681 (7)	0.8 (50)	6.1 (10)	4	384 (37)	3.0 (42)	19.3	0.4 - 29.6
	60	20	12 (60)	590 (36)	2.8 (86)	9.3 (32)	7	407 (20)	5.5 (72)	16.4	0 - 27.6
	61	20	13 (65)	512 (29)	2.9 (59)	9.7 (51)	3	507 (56)	3.6 (80)	18.3	12.7 - 23.1
	69	24	1 (4)	1044	0.5	7.1	19	509 (46)	2.9 (79)	5.2	0 - 39
	69S	62	23 (37)	628 (39)	4.6 (83)	nr	39	529 (48)	nr	nr	
	85S	92	13 (14)	673 (37)	1.2 (52)	nr	79	698 (26)	nr	nr	
25 months:	69S	62	30 (48)	863 (47)	7.5 (57)	nr	32	668 (40)	7.2 (61)	nr	
	85S	87	44 (51)	1238 (34)	3.7 (56)	nr	43	1186 (32)	6.7 (42)	nr	

Family		13 months			15 months			19 months			25 months		
		n	%	cv	n	%	cv	n	%	cv	n	%	cv
35	normal	5	37.9	64.9	8	24.6	109.0	2	0.7	142.8			
	deformed		8.5	95.3		9.4	76.6		6.3	142.9			
60	normal	1	3.1	-	12	5.4	101.9	10	58.8	18.5			
	deformed		8.9	-		48.7	61.6		2.7	85.2			
61	normal		-	-		-	-	11	60.7	19.8			
	deformed		-	-		-	-		3.5	68.8			
69	normal		-	-	2	1.2	141.7		-	-			
	deformed		-	-		29.6	141.2		-	-			
69S	normal		-	-	16	32.8	94.8	23	23.4	120.9	29	30.9	106.0
	deformed		-	-		3.5	103.9		8.1	106.2		6.2	100.6
85S	normal		-	-		-	-	11	10.1	137.6	43	53.1	53.7
	deformed		-	-		-	-		6.3	82.5		8.9	69.8

Table 2: Mean yields (%) of normal and deformed larvae per homozygous family during the experimental period. Percentages are expressed as % of the total number of eggs incubated. n = number of duplicate egg samples. cv = coefficient of variation

4. Egg quality in homozygous gynogenetic families of common carp

From all egg batches obtained, a duplicate egg sample (approx. 200 eggs) was fertilized with a mixed sperm sample of the sperm of 3 gynogenetic males (Komen et al., 1991) and incubated at 24 °C. At 96 hours after fertilization, the percentage of normal and deformed larvae (both expressed as the percentage of the total number of incubated eggs) was determined. Results are shown in table 2. Variation in egg quality was high between and within families. Coefficients of variation within families were 18.5 - 142.8 % for the percentage of normal larvae and 61.6 - 142.9 for the percentage of deformed larvae.

Since animals were individually tagged in family 69S and 85S, egg quality parameters were obtained from individual fish at several samplings. In figure 3, the relation between normal and deformed larvae obtained from individual fish at consecutive samplings is depicted. Regression analysis revealed that in family 69S, egg quality within individuals is relatively constant, especially for the percentage of normal larvae ($R^2 = 0.59$, $P = 0.03$ resp. $R^2 = 0.78$, $P = 0.00$ at 15-19 months (fig. 3B) and 19-25 months (fig. 3D). In family 85S, no significant linear relations were obtained.

The regression coefficient r in these graphs equals the repeatability, and can be regarded as the upper limit for the estimate of h^2 since repeatability is defined as

$$b = \frac{cov\ (x, y)}{var\ (x)} = \frac{V_A + V_{E_g}}{V_P} = repeatability$$

where V_{Eg} = general (permanent) environmental variance.

The repeatability for animals from family 69S was consistently higher than for animals from family 85S. It seems possible to select animals with high egg quality from family 69S, especially on the basis of the percentage of normal larvae obtained.

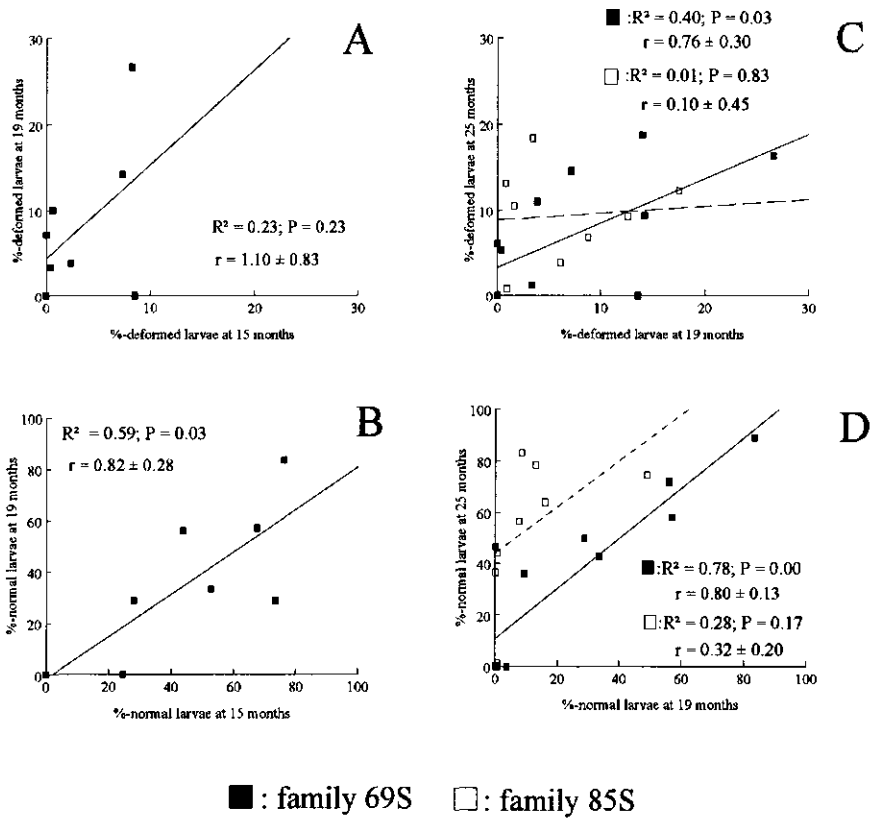

Figure 3.Results of regression analysis for the percentages of normal and deformed larvae for individual fish at consecutive samplings in families 69S and 85S. r = regression coefficient
A: Deformed larvae; 15-19 months; 69S
B: Normal larvae; 15-19 months; 69S.
C: Deformed larvae; 19-25 months; 69S + 85S.
D: Normal larvae; 19-25 months; 69S + 85S.
A P-value < 0.05 indicates a significant linear relation.

5. Estimates of heritability in homozygous gynogenetic families

In estimating heritabilities, female parents should be representative for the additive genetic variation present in the original population. We obtained homozygous families from 5 different females. In producing the homozygous families, genetic variation for egg quality will have been lost since for successful gynogenesis, high quality eggs are needed (Komen et al., 1991; Bongers et al., 1994). No information regarding age at maturation of the female parents was obtained. The analysis was performed only with data obtained during sampling at 13 months since at that moment, no animals had been removed yet from the experiment. However, for the estimates of heritability for egg quality parameters data used were those obtained at 19 months to increase the number of observations (see table 1 and 2). Data were analyzed using the following statistical model

$$\mathbf{Y} = \mathbf{X}\beta + \mathbf{Z}\mathbf{a} + \mathbf{e}$$

where $\mathbf{Y}$ = a vector with phenotypic observations
$\mathbf{X}$ = a matrix relating observations to fixed effects
β = a vector with fixed effects
$\mathbf{Z}$ = a matrix relating observations to individuals
$\mathbf{a}$ = a vector with random genetic effects
$\mathbf{e}$ = a vector with random residual effects

with var($\mathbf{Y}$) = $\mathbf{ZAZ'}\,\sigma_a^2 + \mathbf{I}\,\sigma_e^2$ and where A is a matrix that contains the additive genetic relations between individuals. Individuals included in the analysis were gynogenetically produced individuals with their parents. Phenotypic observations were only available on the offspring. The only fixed effect included in the analysis was the overall mean. Heritability estimates were obtained using Gibbs sampling as described by Wang et al. (1994) and Janss et al. (1995).

Results of the analysis are presented in table 3. The estimated 90% highest posterior density region, the Bayesian equivalent of the confidence interval, indicates that heritability estimates for most traits are not very accurate, but greater than zero. Based on the results of the analysis traits can be categorized into three groups. The first group consists of length, weight, egg weight and pGSI, i.e. traits that seem to have a medium heritability. Group 2 consist of traits with a high heritability, i.e. gonad weight, GSI and % normal larvae, and a third group for traits with an apparent low heritability (not significantly different from zero) that consists of % deformed larvae. Figure 4 gives a graphical representation of the estimated marginal posterior distribution for pGSI (medium heritability), GSI (high heritability) and % deformed larvae (low heritability). These marginal posterior densities suggest that there is additive genetic variance to produce early or late maturing homozygous animals with high egg quality.

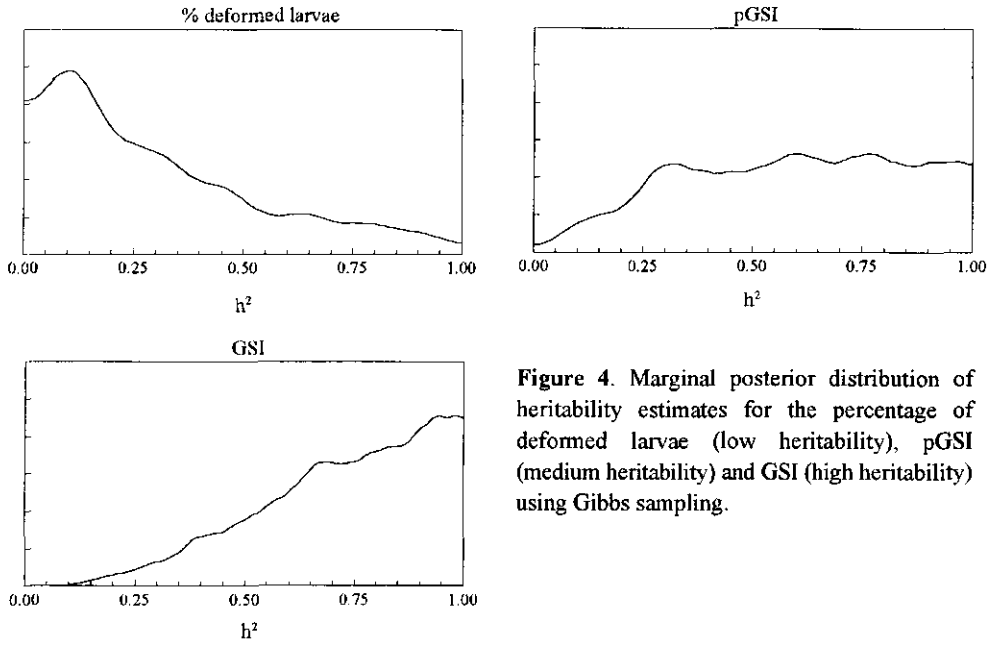

Figure 4. Marginal posterior distribution of heritability estimates for the percentage of deformed larvae (low heritability), pGSI (medium heritability) and GSI (high heritability) using Gibbs sampling.

Table 3. Heritability estimates based on the mean of the marginal posterior distributions with the estimated 90% HPD-region. The Gibbs chain was run with a chain length of 500000. The burn-in period was 50 and a skip parameter of 1000 was used to obtain independent samples from the posterior distribution.

Trait	Mean	90% Highest Posterior Density region
Length	0.496	0.142 - 1.000
Weight	0.580	0.227 - 1.000
Egg weight	0.592	0.262 - 1.000
pGSI	0.583	0.252 - 1.000
Gonad weight	0.752	0.482 - 1.000
GSI	0.722	0.431 - 1.000
% normal larvae	0.727	0.461 - 1.000
% deformed larvae	0.307	0.000 - 0.689

6. Discussion

The additive genetic variance among homozygous populations is doubled compared to the additive genetic variance in the base population. Without giving formal derivation, Quillet (1994) stated that additive genetic variance within a homozygous gynogenetic family is half the original additive genetic variance and between family additive genetic variance then becomes 1½ times the original additive genetic variance. However, we derived that the distribution of additive genetic variance between- and within gynogenetic families has a 1:1 ratio. As a result, in studies where gynogenetic offspring from only one female is compared with normal diploid offspring (Sumantadinata et al., 1990; Komen et al., 1992; Hussain et al, 1995), an increase in phenotypic variance results from increased environmental variance, due to an increase in susceptibility of homozygous individuals to the environment. Further, we found evidence that in gynogenesis, the shock treatment per se induces a large amount of additional phenotypic variation (embryonic damage: Bongers et al., 1996). For these reasons, heritability estimates (σ_A^2/σ_P^2) from gynogenetic or androgenetic studies might be different from the ones found in studies using normal reproduction. Also, an unknown fraction of the gynogenetically produced fish might not survive due to homozygosity at some deleterious loci. This leads to loss of additive genetic variance and, as a result, to different heritability estimates.

In the rainbow trout, *Oncorhynchus mykiss* Gjerde and Gjedrem (1983) estimated heritabilities from sib analysis for age at maturation of 0.18 ± 0.07 and 0.09 ± 0.05 (sire resp. dam component of variance). In the Atlantic salmon, *Salmo salar* they found values of 0.42 ± 0.08 resp. 0.15 ± 0.03. Naevdal et al. (1976) estimated values of 1.00 resp. 0.67 (no s.d. presented) in this species. For the trait "dead eyed eggs", Kanis et al. (1976) estimated heritability using half sib analysis in *O. mykiss* and *S. salar*. They found values of 0.15 ± 0.04 and 0.27 ± 0.04 resp. 0.05 ± 0.04 and 0.62 ± 0.05. In laboratory populations of *O. niloticus*, Kronert et al. (1989) estimated heritabilities for maturation of females to be 0.48 ± 0.12. For the parameters GSI and %-normal larvae we estimated heritabilities in homozygous gynogenetic families of 0.72 (90 %-HPD region = 0.43 - 1.00) resp. 0.73 (0.46 - 1.00). As mentioned earlier, heritability estimates from gynogenetic families might differ from those obtained from conventional families. Nevertheless, we show that additive genetic variance can be estimated in homozygous families. The trait %-normal larvae is of particular importance for the maintenance of important homozygous genotypes.

Homozygous animals are thought to be more susceptible to the environment than outbred animals (Falconer, 1989). Egg quality can be affected by maternal influences. As a result, in homozygous families, changes in the environment can cause an increased maternal influence on egg quality (Kincaid, 1983). Family 69S showed a constant egg quality per individual during the

course of the experiment, thereby revealing the possibility for selecting homozygous fish with high egg quality.

We removed phenodeviants from the families at the age of 6 months. Phenodeviants occupy an intermediate position between qualitative and quantitative traits and do not lend themselves to genetic analysis (Kirpichnikov, 1981). We recently found that phenodeviants can result from embryonic damage, generated during the heat shock treatment (Bongers et al., 1996). Therefore, removal of the phenodeviants should not be regarded as genetic selection in the experimental population.

In the platyfish, *Xiphophorus maculatus* a sex linked gene P determines the age at which maturation occurs. Five P alleles have been identified (P_1 through P_5). Development of the reproductive system is characterized by the successive appearance of immunoreactive GnRH in the brain, the proliferation of gonadotrophs in the pituitary and the maturation of the gonads (Schreibman et al., 1989). Genetics of maturation in platyfish and common carp show similarities (Kallman and Borkoski, 1978; Hulata et al., 1985). Early maturing genotypes are in general small and highly fecund whereas late maturing genotypes are larger but less fecund. Animals from the late maturing family 85S were significantly larger and had lower pGSI's than fish from the earlier maturing family 69S. These two families were grown in the same tank, so had common environments. In common carp culture, late maturation is preferred since a negative correlation exists between growth rate and onset of sexual maturation (Hulata et al., 1985). In *O. niloticus* where late maturation is preferred to prevent stunting, no genetic correlation was found between onset of female maturation and growth (Kronert et al., 1989). Sex hormones are thought to interfere with growth hormones and depress growth (Kallman and Schreibman, 1973; Johnstone et al, 1979; Hulata et al., 1985). However, Joblin (1994) states that sex steroids in general have a growth promoting effect and that the decrease in growth in maturing animals is an effect of the competition between somatic and reproductive growth. However, within families, mature animals were always larger than their non-mature siblings (table 1).

Gynogenesis and androgenesis can be useful additional tools in selection programs. It can be used to estimate directly the additive genetic variance of a certain trait and the breeding value of individual parents, revealing the possibility for improving stocks by selection. For estimating heritabilities approximate formulas can be derived to obtain the optimal size of a gynogenetic family and the sampling variance of the heritability estimates (Bijma et al., 1996). The optimal number of gynogenetic progeny per family than equals $2 + 1/h^2$, whereas in a half sib design the optimal number of progeny is $1 + 4/h^2$. These formulas show that the optimal family size in a

gynogenetic family is always smaller than in a half sib family. The approximate sampling variance of our heritability estimates is

$$\sigma^2_{h^2} = \frac{8\,h^2\,(h^2+1)}{T}$$

where T is the total number of individuals in the experiment, i.e. number of families times number of gynogenetic progeny per family. This sampling variance is only lower than in a half sib design when $h^2 < 0.35$ (Bijma et al., 1996). Reducing the number of animals to be analyzed using gynogenetic progeny is advantageous, especially when the analyses are laborous and/or costly. Wether this advantage is undone by the negative aspects of a gynogenetic family structure (increased environmental variance, possible loss of additive genetic variance) needs further investigation.

At this moment, we have selected several early and late maturing animals from families 69S and 85S with high egg quality. Homozygous clones have been produced from these females by gynogenesis. Examination of age at maturation of these clones will give more information on the genetic background of this important trait in common carp.

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Chapter 8

Genetic analysis of testis development in all-male F1 hybrid strains of common carp, *Cyprinus carpio*

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Abstract
Genetics of testis development was examined in 12 all-male F1 hybrid strains of common carp, *Cyprinus carpio*, produced after crossing four homozygous androgenetic males with three gynogenetic females. These parents were selected for fast or slow gonad development. In the F1 hybrids, additive genetic and dominance and interaction variance was examined by regular sampling between the age of 100 and 240 days of age. The onset of spermatogenesis, ranging from 100 - 130 days after hatching was controlled mainly by additive genetic effects. Testis somatic index (TSI) ranged from 2.0 - 5.5 % at the end of the experiment. Early onset of spermatogenesis coincided with high TSI and late onset of spermatogenesis with low TSI. In the experiment, the female parent affected TSI to a greater extent than the male parent. For this parameter additive genetic effects also accounted for most of the variance observed. These data fit to a great extent with the established genetic model for gonadal development in the platyfish, *Xiphophorus maculatus*. From analysis of within-strain variations, we conclude that F1 hybrids are suitable as experimental animal models.

1. Introduction

Age at first maturation is an important character in fish breeding (Kirpichnikov, 1981). The biology of (early) gonadal development is extensively studied in many fish species (Nagahama, 1983; Yaron, 1995). In general, gonadal growth is initiated after activation of the brain-pituitary-gonad (bpg) axis. Releasing hormones (GnRH) from the hypothalamic region induce the release of gonadotropic hormones (GTH) from the pituitary. In pubertal fish, GTH induces spermatogenesis or oogenesis through local production of resp. androgens and estrogens. In contrast, genetics of gonad development is still relatively poorly understood. Schreibmann et al. (1989) reviewed literature on genetic influences on reproductive system development and showed that genetic variance exists within various marine and freshwater species. Comprehensive studies of the platyfish, *Xiphophorus maculatus* revealed that in this species, a sex linked gene P determines the age at which sexual maturation occurs. At least 5 alleles were identified. It has been hypothesized that the P-gene might be the "switch" to activate the bpg-axis, or that it regulates the fate of GnRH's (Kallman and Borkoski, 1978). Hulata et al. (1974) found genetic differences in gonad development between several races of (slowly developing) european and (fast developing) chinese common carp, *Cyprinus carpio*. Crossbreeding experiments yielded dominance effects, where within the european races fast development was dominant over slow development. When chinese carps were crossed to european carps, incomplete dominance in the direction of the european parent (slow development dominant over fast development) was observed (Hulata et al., 1985).

We recently showed from several homozygous gynogenetic families that a considerable amount of additive genetic variance exists for gonad development in common carp. We estimated h^2 = 0.72 for the gonado-somatic index at the age of 13 months (Bongers et al., 1996a). In homozygous populations, dominance variance is thought to be absent (Falconer and Mackay, 1996). To determine the relative importance of dominance and additive genetic effects, the

following experiment was performed. Twelve all-male (XY) F1 hybrids were produced by crossing four homozygous androgenetic (YY) males (Bongers et al., 1996b) with three gynogenetic (XX) females in a complete diallel design. Female and male parents were selected for fast or slow gonad development, attempting to create differences in the pattern of gonad development in the F1 hybrids. Examination of variance components (additive resp. dominance and interaction genetic variance) for onset of spermatogenesis and subsequent gonadal growth was done by studying line (F1 hybrid) resp. interaction effects.

2. Material and methods

Selection of homozygous parents

Homozygous females originated from 2 gynogenetic families, designated family 69 and 85, and homozygous males from 2 androgenetic families, designated family A1 and A2. The parents of these four families were full sibs, obtained after crossing Polish and Hungarian carps. Selection of the homozygous females was performed on the basis of their (genetically identical) gynogenetic offspring. To attempt to create a large selection differential, only females that had displayed large differences in age at maturation, i.e. the age at which they ovulated for the first time after hypophysation, were gynogenetically reproduced according to the method of Komen et al. (1991). In family 69, females were considered to be early maturing resp. late maturing when ovulation could be induced at the age of 15 resp. 19-25 months whereas in family 85 this was 19 resp. 25 months (T = 25 °C) (for more details, see Bongers et al., 1996a). In total, eight inbred strains were produced and raised under identical laboratory conditions. They were regularly sampled to determine gonadal growth and the onset of vitellogenesis between 6 and 13 months of age. This procedure resulted in the selection of female 85-29 (fast development), 69-26 (slow development) and 69-82 (intermediate development). Males were also selected for age at maturation, i.e. the age at which sperm became apparent for the first time after gentle abdominal pressure without prior hypophysation. Males were considered to be early resp. late maturing at the age of 150 resp. 240 days (T = 25 °C). Two early (A1E, A2E) and two late (A1L, A2L) maturing males were selected and crossed with the three females to produce the 12 F1 hybrids. The coefficient of inbreeding of the F1 hybrids can be calculated as 0.25 (Bongers et al., 1996a).

Raising of fish

During the first 4 weeks after hatching, 200 larvae per F1 hybrid were raised in 25 l aquaria. They were fed freshly hatched *Artemia* nauplii *ad libitum*. Then, 120 random fish per strain were equally divided over two 70 l aquaria in another recirculation system. The strains were randomly

divided over the 24 aquaria. They were fed vitamin C-enriched trout pellets (Trouw, Vervins, France) at a daily ration of 25 gr per $kg^{0.8}$, based on an overall-mean body weight ($kg^{0.8}$ refers to metabolic body weight; a value of 0.8 was chosen for the weight exponent cf (Winberg, 1956; Heinsbroek, 1989)). Food, daily adjusted according to the expected growth and observed mortality, was administered using Scharfflinger conveyer belt feeders, approx. 12 hours a day. Untill the moment of first sampling, all animals were counted and weighed every four weeks. Food was gradually reduced to prevent low oxygen concentrations in the aquaria (from 12 weeks onwards: 20 gr per $kg^{0.8}$, 15 weeks: 15 gr per $kg^{0.8}$, 17 weeks: 2 % of the total biomass per day, 26 weeks: 1½ %). Temperature was constantly kept at 25 °C. Because of these standardized laboratory conditions, it is assumed that all strains have been subjected to common environmental effects.

Parameters recorded and statistical analysis

All strains were sampled at the age of 100, 121, 142, 163, 184, 205 and 240 days after hatching (sample 1 to 7). Five random animals were sacrificed per aquarium and BW, gonadal sex, testis weight (TW, 0.01 g) and TSI ((TW/BW) x 100%) was determined. For each parameter, the within-aquarium coefficient of variation (standard deviation, expressed as percentage of the mean) was calculated at each sampling. At 240 days, all remaining animals were sacrificed (avarage: 26 animals per aquarium). Increase in TSI during the experimental period was plotted for each aquarium and sigmoïd growth-curves were fitted. Onset of spermatogenesis was then estimated as the intersection of the curve with the x-axis (TSI = 0). Data were analyzed using the following statistical model:

$$Y_{ijk} = \mu + \alpha_j + \beta_k + \alpha_j\beta_k + \varepsilon_{ijk} \qquad \text{where}$$

Y_{ijk} = observation (parameter-mean of five individuals per aquarium, i = 1,2)
μ = overall mean
α_j = main effect of the male parent (j = 1,2,3,4)
β_k = main effect of the female parent (k = 1,2,3)
$\alpha_j\beta_k$ = interaction between male and female parent
ε_{ij} = experimental error

All calculations were performed with aquarium-means. We do not consider the five fish per aquarium as independent samples, due to interactions between individual fish from the same aquarium. Therefore, the aquaria are considered to be the experimental units. For determination

of main effects, parental and interaction effects were considered to be fixed effects. For the estimation of variance components of main and interaction effects (i.e., variance of general and specific combining abilities: Falconer and Mackay, 1996) parental and interaction effects were considered to be random effects. They were calculated using ANOVA (restricted maximum likelihood estimates: REML) and related to resp. additive genetic effects and dominance and interaction effects. All analyses were performed using BMDP computer programs (Dixon et al., 1988).

3. Results

Growth of all F1-hybrids was equal during the experiment. Mean body weights per strain increased from 32.1-51.2 g at the first sample to 175.3-209.4 g at the last sample. No significant parental effects were detected concerning growth. In contrast, testis development varied greatly between the strains. Figure 1 shows testis development for the fastest (A2E × 69-26) and the slowest (A1L × 85-29) developing strain.

The onset of spermatogenesis varied between approx. 100 to 130 days after hatching (table 1). From the general combining abilities in this table it can be concluded that female 69-26 carries genes for early, female 69-82 for avarage and female 85-29 for late(r) onset of spermatogenesis. The general combining abilities for male parents show that males from family A2 carry genes for earlier onset of spermatogenesis than males from family A1. Analysis of variance using REML to estimate variance components yielded that male parents accounted for 49 % of the total variance. The female, interaction and error component accounted for resp. 22, 10 and 19 % of the total variance. This suggests the existence of a considerable amount (49 + 22 = 71 %) of additive genetic variance.

Parental effects were also found for TSI. Table 2 shows TSI in all hybrids at the last sampling (240 days). Females 69-26 and 69-82 yielded offspring with higher TSI than female 85-29. Also, males from family A2 yielded offspring with higher TSI than males from family A1.

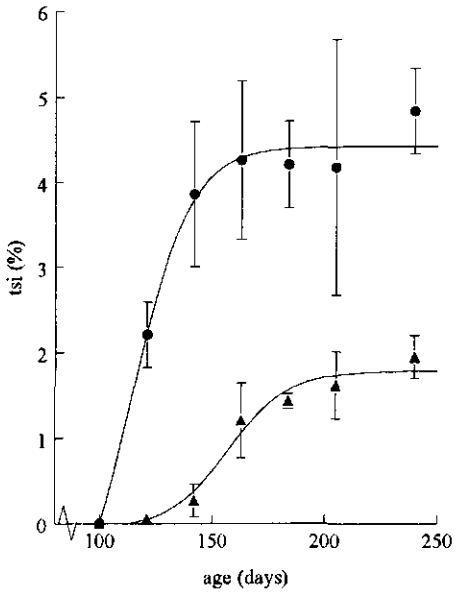

Figure 1: Testis development of the fastest (●: A2E x 69-26) and the slowest (▲: A1L x 85-29) developing F1 hybrid strain. TSI = Testis Somatic Index. Each point represents the mean of the duplicate aquaria; bars represent standard deviation.

Table 1: Estimates of onset of spermatogenesis (days after hatching) for every combination of parents per aquarium. General combing abilities (GCA, main effects parents) are calculated as parental mean minus overall mean μ. P-(male effect): 0.000, P-(female effect): 0.002, P-(interaction): 0.13

male parents: female parents:	A1E	A1L	A2E	A2L	mean	GCA
69-26	120 119	117 119	100 99	114 114	112.8	-4.2
69-82	122 122	116 120	115 109	117 111	116.5	-0.5
85-29	131 125	131 124	114 118	121 109	121.6	4.6
mean	123.2	121.2	109.2	114.3	μ = 117.0	
GCA	6.2	4.2	-7.8	-2.7		

At sample 1, testis development was undetectable in all strains. Analysis of variance components for sample 2 to sample 7 are summarized in table 3. It can be read that at these samplings, the female component was consistently found to be larger than the male component. Only at sample 2, a large interaction component was found (60.5 %). However, this was due to the fact that at this sampling only two strains had started testis development (A2E x 69-26 and A2L x 69-26; data not shown).

Table 2: Testis somatic index (TSI) for every combination of parents per aquarium at the last sampling (age: 240 days). General combing abilities (GCA, main effects parents) are calculated as parental mean minus overall mean μ. P-(male effect): 0.002, P-(female effect): 0.000, P-(interaction): 0.09.
-[1]: aquaria were lost due to technical failure between sample 6 and 7.

male parents: / female parents:	A1E	A1L	A2E	A2L	mean	GCA
69-26	3.40 3.59	3.66 3.71	4.48 5.19	4.63 -[1]	4.09	0.48
69-82	2.25 2.94	3.25 5.08	5.27 4.11	5.94 5.28	4.27	0.66
85-29	1.65 -[1]	2.12 2.01	3.50 3.52	2.12 1.77	2.38	-1.23
mean	2.77	3.31	4.35	3.95	μ = 3.61	
GCA	-0.84	-0.30	0.74	0.34		

Table 3: Partitioning the total phenotypic variance (σ^2_P) for the parameter tsi into parental (male and female), interaction and environmental components of variance for sample 2 to sample 7. At sample 1, no testis development was observed yet in any of the strains. Components of variance are displayed as percentages of σ^2_P. Parental components can be considered as the variances of the general combining abilities, and the interaction component as the variance of specific combining abilities.

	mean tsi	σ^2_P	σ^2-♂	σ^2-♀	σ^2-♂*♀	σ^2_e
sample 2	0.44	0.43	11.6	18.6	60.5	9.3
sample 3	1.78	2.08	14.9	56.7	4.8	23.6
sample 4	2.35	2.26	27.4	33.2	14.6	24.8
sample 5	2.63	1.87	30.5	31.6	16.6	21.4
sample 6	3.22	2.15	20.9	53.5	10.7	14.9
sample 7	3.61	1.80	22.8	43.3	16.7	17.2

During the total experimental period, the coefficient of variation (cv) in the F1 hybrids for the parameter body weight ranged from 22 - 34 %. Much higher cv's were observed for TSI (15 - 74 %). However, clear relations were found between relative testis size and the cv. Figure 2 shows results of this linear regression analysis at sample 6. At high TSI (5-6 %), cv was found to be as low as 15-25 %.

When combining data from table 1 and 2, it can be found that early onset of spermatogenesis coincides with high TSI at the last sample, and vice versa. This negative correlation was confirmed by linear regression analysis: $R^2 = 0.31$; $P = 0.007$.

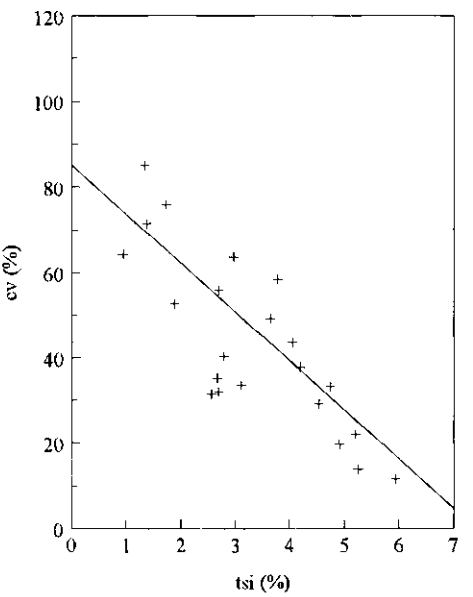

Figure 2: Illustration of the negative correlation between the testis-somatic index (TSI) and the coefficient of variation (cv) for this parameter. Data are derived from the sixth sample (205 days). Each cross represents an observation from one aquarium.

4. Discussion

Homozygous male and female parents were selected for age at maturation. This resulted in all-male strains with genetically different patterns of onset of spermatogenesis (range: approx. 100 - 130 days) and TSI (2.0 - 5.5 % at 240 days). Differences in pattern of testis development can only be attributed to genetic factors since rearing conditions were highly standardized, so all strains were subjected to a common environment. We found indications for the existence of additive genetic variance for the onset of spermatogenesis and subsequent testis development in our common carp strains. The female parent largely determined the TSI (table 3). Dominance and interaction effects were not detected. In the platyfish, *Xiphophorus maculatus*, Kallman and Borkoski (1978) also found that the alleles determining onset of gonad development acted largely in an additive way. However, interactions between specific allele-combinations were also documented. Hulata et al. (1974) found dominance effects for gonad development in european races of common carp, based on relative gonad weights in males and females at the age of 7 months. These authors determined dominance effects from deviations of five crossbreds from the mid-points of the parents. Fast development was found to be dominant over slow development. These differences between our study and the study from Hulata et al. 1974) can be caused by the different method of determining dominance effects, or by the fact that in our study all parents came from the same pedigree.

Kallman and Borkoski (1978) and Hulata et al. (1985) stated that similarities existed between patterns of gonadal development in *Xiphophorus maculatus* and *Cyprinus carpio*. Early maturing genotypes are in general more fecund than late(r) maturing genotypes. We present a similar relation between onset of spermatogenesis and fecundity in terms of TSI. However, the present experiment was terminated at 240 days. It is not clear whether the TSI of slowly developing strains would have continued to increase (see f.e. strain A1L x 85-29 in figure 1). Kallman and Borkoski (1978) and Hulata et al. (1974, 1985) also found that, as adults, late maturing genotypes were heavier than early maturing genotypes. We did not observe these differences in body weight. However, this can be due to the feeding strategy, which was based on an overall mean body weight.

The homozygous females were selected on the basis of their gynogenetic (genetically identical) offspring. Female 69-26 was selected as a slowly developing and female 85-29 as a fast developing genotype. In the gynogenetic offsprings of these females, estimation of the onset of vitellogenesis resulted in 268 days resp 235 days after hatching (data not shown). Estimation was performed comparably as for the onset of spermatogenesis in the present experiment. However, we found that female 69-26 sired the earliest maturing strains whereas female 85-29 the latest maturing strains (table 1). This suggests that alleles or genes for onset of ovary development and

testis development in common carp are not (always ?) comparable. In the platyfish, the allele for late maturation in females is linked to the X-chromosome (Kallman and Borkoski, 1978).

We analyzed genetics of gonadal development in standardized strains of common carp. Standardized strains can 1) increase the replicability (= lower variation between replicates during a single measurement), 2) increase repeatability (= lower variation between tests within the same laboratory) and 3) increase reproducability (= lower variation between tests from different laboratories) (Dave, 1993). Replicability was high in the present experiment, as can be seen from the duplicates in table 1 and 2. Variation in body weight remained constant during the experiment (cv: 22 - 34 %). For F1 hybrids, we consider this variation rather high since in other studies from our laboratory, we measured variations in F1 hybrids for body weight already as low as 11 -16 % (Komen et al., 1993). For TSI we found a relation between absolute TSI and the variation (fig 2). When TSI reaches maximum values (5-6 %) variations observed were minimal (15 - 25 %).

Inbred strains are widely used in many areas of (mammalian) biological research when genetic uniformity is desired. However, in many types of research it is phenotypic uniformity that is of primary interest. In general, F1 hybrids are more viable, healthier and more fertile than inbred strains. F1 hybrids combine both genetic and phenotypic uniformity and can thus reduce the number of animals used in experimental animal research (Festing, 1979; Falconer and Mackay, 1996). We show that when F1 hybrids are used, limited numbers of fish are sufficient to obtain significant results in the genetic analysis of a complex trait as gonad development.

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Chapter 9

General discussion

This thesis describes the development and application of genetically uniform carp strains. The present chapter is divided in the production of genetically uniform fish strains, the applications of genetically uniform strains for fundamental and applied research and the monitoring of genetic quality control (i.e., testing genetic purity of genetically uniform strains).

1. Production of genetically uniform fish strains

The development of genetically uniform fish strains consists of the production of homozygous larvae and subsequently raising these larvae to fertile broodstock. Problems associated with these two steps are high mortality during the production of homozygous larvae and reduced fitness and fertility of homozygous individuals.

Total mortality after the treatment to induce homozygosity is high, in general 90 - 95 % (Streisinger et al., 1981; Naruse et al., 1985; Scheerer et al., 1986, 1991; Quillet, 1994; Komen et al., 1991; Taniguchi et al., 1991; Bongers et al., chapter 3, this thesis; Johnstone and Stet, 1995). The treatment consists of two parts: (1) elimination of the genetic contribution of one of the parents by irradiating gametes and (2) doubling of the haploid genome by physical shocks. The irradiation procedure is optimal when the DNA is fully destroyed without affecting the basic function of the gamete (i.e., fertilizing capacity for sperm; serving as "embryo-host" for eggs). In gynogenesis, the sperm irradiation treatment does not have a large impact on mortality since in meiotic gynogenesis (retention of the second polar body: "heterozygous gynogenesis") survival is in general high (in carp, *Cyprinus carpio*: 56 %, Komen et al, 1988; in african catfish, *Clarias gariepinus*: 81 %, Volckaert et al., 1994; in atlantic salmon, *Salmo salar*: 95 %, Johnstone and Stet, 1995). In androgenesis, UV-irradiating eggs seems to do little damage beyond nuclear inactivation since androgenesis and gynogenesis yield similar amounts of haploids in rainbow trout, *Oncorhynchus mykiss* (Parsons and Thorgaard, 1984) and in carp (Komen and Bongers, unpublished results).

Several studies provide evidence for the destructive effect of the shock treatment (e.g., Chourrout et al., 1986, Komen et al., 1991). The most convincing evidence was obtained by Thorgaard et al. (1990). They fertilized irradiated rainbow trout eggs from the same batch with (normal) haploid sperm from diploid males, followed by a heat shock and with diploid sperm from tetraploid males without subsequent heat shock. Survival of diploids generated from tetraploid males was much higher than from diploid males (mean relative survival: 42.8 vs 0.8 %). There is however no general effect of the shock treatment, since its effect depends on the timing. When shocks are applied too early, high percentages of haploids hatch (for example, chapter 5: up to 35.8 %) while shocking too late results in total mortality (for example, Komen et al., 1991). The treatment is only efficient in inducing diploidy at the metaphase of the first mitotic division. Due

to variation in developmental rate between eggs from the same batch, only a fraction of the eggs are in metaphase when the treatment is applied. In gynogenesis to retain the second polar body, the treatment is applied shortly after fertilization. At that moment, all eggs are in metaphase of the second meiotic division and yields of gynogenetic diploids is therefore in general much higher than after homozygous gynogenesis (Komen et al., 1991).

Recessive deleterious genes are of minor importance as a cause for mortality, as shown in studies where homozygous males (free of recessive deleterious genes) yielded equal amounts of androgenetic diploids as outbred males (in rainbow trout: Scheerer et al., 1986, 1991; in carp: Bongers et al., chapters 3 and 5, this thesis). In gynogenesis, inferior egg quality of homozygous females (i.e., not resistant to the heat shock: Komen et al., 1991; Quillet et al., 1994) inhibits comparisons of gynogenetic yields from homozygous females with outbred females.

For several reasons, only a small proportion of the initial number of homozygous larvae will develop into fertile broodstock. Firstly, the chromosome manipulation treatment induces deformations in androgenetic or gynogenetic offsprings (embryonic damage: chapter 6). Therefore, during the growth period animals are being discarded. Secondly, reduced fitness due to inbreeding depression is assumed to result in relative high mortality observed during the growth period in homozygous families compared to normal (outbred) families (Komen et al., 1991). Thirdly, inbreeding depression frequently affects fertility to a large extent (Falconer and Mackay, 1996). For example, Komen et al. (1991) stated that less than 10 % of the homozygous carp females within one gynogenetic family could be reproduced gynogenetically to produce inbred strains. In this thesis we attempted to produce homozygous gynogenetic inbred strains from a total of 26 females (chapter 8). These females were selected from two gynogenetic families (family 69 and 85; initial number of females 190: chapter 7). Eight females yielded substantial amounts of gynogenetic (clonal) offsprings, representing only 4.2 % of the initial number of females.

The consequences of these limitations concerning the production of gynogenetic inbred strains can best be illustrated with an example: when 25 gynogenetic inbred strains are desired, approximately 15.000 eggs have to be treated assuming that ± 5 % survives the treatment (750 left), ± 10 % is discarded due to severe deformations (675 left), ± 25 % is lost due to mortality in general (500 left) and ± 5 % is fully fertile (25 left). This suggests that gynogenetic inbred strains can only be developed in fish species with high fecundity. However, females can be selected with high egg quality to improve the yield of gynogenetic offspring since in chapter 7 is was shown that additive genetic variance for high egg quality is present. Also, different egg batches showed to be differently susceptible to the treatment (chapter 5 and 6), so not only for survival but also for the "quality" of the offspring females can be selected. Wiegertjes (1995)

showed that within homozygous carp families genetic differences existed in disease resistance, indicating the possibility to select disease resistant genotypes to reduce mortality during the growth phase.

Reduction of fertility in androgenetic males has no profound effects on the production of androgenetic all-male inbred strains. In chapter 8 the selection of homozygous androgenetic males was described. These males were selected from two androgenetic families, produced from outbred male parents. In both families, approx. 20 % of the total number of animals showed to be mature males after checking for running milt at the age of seven months (data not shown in chapter 8). This percentage represents nearly half the amount of male (YY) genotypes in androgenetic families.

2. Applications of genetically uniform carp strains

For qualitative traits, individuals can be assigned to distinct classes. The mechanism of inheritance is analyzed by segregation patterns of genotypes into Mendelian ratios. Quantitative traits are influenced by many loci and therefore individual genes cannot be identified by their segregation into Mendelian ratios (Falconer and Mackay, 1996). Further, quantitative traits are usually influenced by environmental factors (Hartl and Clark, 1989). Analysis of quantitative (polygenic) traits requires statistical methods which use the degree of resemblance between relatives to determine modes of inheritance. These methods test whether the total (phenotypic) variance can be partitioned into a (significant) genetic component and an environmental component.

Gynogenetic or androgenetic reproduction yields offsprings with genetic relations uncommon in regular quantitative genetics. Therefore a quantitative genetic framework was developed and Gibbs sampling was used to estimate variance components (chapter 7). As stated previously, for the production of gynogenetic inbred strains homozygous females with high egg quality are needed. Several gynogenetic families were produced and genetic variance was estimated for the parameters egg quality and age at maturation. For both parameters, it was found that genetic variance differed significantly from 0. It was indicated that gynogenesis and androgenesis can potentially be useful additional tools in selection programs. Bijma et al. (1996) studied the use of gynogenesis or androgenesis in order to estimate breeding values and variance components in more detail. They showed that in a gynogenetic or androgenetic family structure the optimal family size is always smaller than in a full sib or half sib family structure. However, the accuracy of the heritability estimate from gynogenetic or androgenetic families is only higher at low heritability (< 0.35). Nevertheless, it is stressed that gynogenesis and androgenesis are not alternatives to classical genetic improvement programs, the main reason being loss of fertility

in inbred animals. Laboratory-scale experiments with gynogenetic or androgenetic animals can be used as information source for the traits under selection. For example, our findings in chapter 8 showed that economic important traits can be analyzed using F1 hybrids. Small numbers of fish (10 per F1 hybrid per sampling) were sufficient to obtain significant results. The production of androgenetic or gynogenetic clones facilitates measurement of different traits on the same genotype, useful for estimating genetic correlations (Bijma et al., 1996). In the former USSR, gynogenesis is already used in selection programs of common carp, however only to map genes and ".....to facilitate genetic investigations" (Kirpichnikov, 1987).

In bioassays (mainly toxicity tests), Cypriniforms are used in at least 25 % of all studies and common carp is the most frequently used among the Cypriniforms (chapter 2). However, research on teleosts is frequently performed on fish with unknown genetic background and raising history. In cooperation with the Laboratory for Biological Research in Aquatic Pollution, Ghent and the Laboratory for Ecology and Aquaculture, Leuven, Belgium preliminary toxicological assays were performed with cadmium as toxicant. The aim of these assays was to demonstrate that with genetically uniform carp strains experiments with high replicability, repeatability and reproducability could be performed. Therefore, 96 h static bioassays were conducted in February and May 1996 to determine LC50 values of cadmium. Tests were performed in duplicate using an outbred strain and an inbred strain of common carp, and conducted at the two laboratories at the same time. The experiment was repeated with the same inbred strain, and with a different outbred strain. Repeatability and reproducability were high when using the inbred strain (table 1). Repeatability was low for the outbred strain. High mortality in the second assay was already observed in the lowest concentrations, and no LC50 could be calculated. These preliminary results emphasize the importance of using genetically uniform fish in bioassays. In other toxicity-studies on cadmium in fish, Hwang et al. (1995) obtained several tilapia broods from a fisheries research institute. Wicklund Glynn et al. (1994) obtained zebra fish from the local pet shop. In both papers high variation within experiments was observed, resulting in restrictions regarding statistical comparisons. Genetic differences between broods resp. individual fish were suggested to be the cause of this variation.

Table 1: 96-h LC50 values (95 % confidence intervals) for cadmium (as $CdCl_2$) in two separate bioassays, determined and repeated at two laboratories at the same time. Assays were performed according to OECD guidelines. In short, tests were performed at 21-23 °C at a photoperiod of 12 h light : 12 h dark in 2 l glass jars. Nominal $CdCl_2$-concentrations were 0, 2, 4, 6,.....16, 18 mg per l. Per concentration, seven fish were tested in duplicate. The inbred carp strain was obtained after crossing homozygous siblings of the congenic inbred strain E4-*X/Y*-5 (see chapter 4). The outbred strain in bioassay 1 was obtained after crossing partly outbred animals from a Dutch carp strain. The outbred strain in bioassay 2 was produced by crossing a similar female with outbred males originating from Poland. At the start of both bioassays, fish were 5 weeks old. LC50 values were calculated using the Moving Average method. * : no LC50 calculated due to high mortality in lowest concentration.

	inbred strain		outbred strain	
	assay 1	assay 2	assay 1	assay 2
Ghent	6.47 (5.43-7.43)	3.53 (2.24-4.60)	6.68 (5.69-7.64)	- *
Leuven	5.27 (4.26-6.19)	5.35 (4.45-6.19)	6.65 (5.26-7.97)	- *

3. Genetic quality control

As shown above, the genetic background of animals can seriously affect the outcome of bioassays. Genetically uniform strains should constantly be monitored to check the purity of the strains. Several methods exist for testing the uniformity of inbred strains including skin grafting, biochemical methods (protein variants) or DNA-analysis (DNA fingerprints, RFLP's, microsatellite markers) (Van Zutphen, 1993).

During the production of inbred strains of carp, several controls were performed (1) to prove absence of one of the parental genomes, (2) to prove homozygosity in gynogenetic or androgenetic offspring and (3) to prove isogenicity within inbred strains. To prove absence of one of the parental genomes, every androgenesis or gynogenesis experiment contained a haploid control to determine the efficiency of the irradiation procedure. In these control groups, no heat shock was administered after initiation of haploid development. The amount of (biparental) diploids hatched is then a measure for the effectiveness of the irradiation treatment. Elimination of one parental genome was further checked by the use of dominant genetic markers. In gynogenesis, we used irradiated sperm of scaled males (dominant trait) to initiate development of eggs obtained from mirror females (recessive trait) (Komen et al., 1988, 1991; Bongers et al., chapter 6, chapter 7). In androgenesis, we used irradiated eggs from black females (dominant trait) as a host for genomes from blond males (recessive trait) (chapter 3 and 5). Since Chourrout (1984) showed that ionizing radiation (X- or γ-rays) could induce transmission of chromosome fragments, UV-irradiation is generally accepted as the method of choice. Ihssen et al (1990)

stated that "for the genetic inactivation of fish eggs, UV irradiation is not effective because of its low penetrating power". However, we show in chapter 3 that in carp eggs UV irradiation while stirring in an synthetic ovarian fluid can be applied to safely eliminate the female genome without risking contamination of androgenetic genomes with maternal DNA fragments.

Homozygosity is frequently confirmed by electrophoretic analysis of protein variants (f.e. in zebra fish, *Brachydanio rerio*: Streisinger et al., 1981; in rainbow trout: Quillet et al., 1991; Scheerer et al., 1991; Quillet, 1994). In carp, Komen et al. (1991) used Mendelian segregation patterns of recessive mutant pigment genes to demonstrate the homozygous nature of gynogenetic offspring. Heterozygous black females (b1,+/b1,b2, +,b2/b1,b2 and +,+/b1,b2) were gynogenetically reproduced using sperm from wild type males. Incidences of blond fry (b1,b2/b1,b2) were resp. 51.7, 51.3 and 27.7 %. A similar Mendelian segregation pattern was observed when the gynogenetic family 69 was produced (chapter 7). Among her gynogenetic offspring, 50 % mirror and 50 % nude (completely scaleless) carps were found, indicating that female 69 was heterozygous for this trait. In androgenesis, we used males heterozygous for the trait scalation (S/s; chapter 4). This resulted in 50 % scaled (S/S) and 50 % mirror androgenetic individuals (s/s).

All controls for elimination of parental genomes and for demonstration of homozygosity used in the present thesis are summarized in table 2. In androgenesis, varying (small) amounts of biparental diploids hatched in the haploid control for egg irradiation (irradiation of eggs without further heat shock). However, in heat shocked groups biparental diploids were never observed when the heat shock was properly timed. In that case, the diploid (biparental) genome is doubled resulting in tetraploid embryos which are not viable (Komen et al., 1991). When gynogenetic families were produced for quantitative genetic analysis of gonad development and egg quality (chapter 7), high numbers of diploid individuals were observed in the irradiation control group of female 85 (data not shown). Raising of these larvae revealed that they originated from spontaneous diploidization since the genetic marker showed absence of male inheritance. When inbred strains were produced to select female genotypes with slow or fast gonadal development (chapter 8), this phenomenon was also observed in four out of nine irradiation control groups. Spontaneous diploidization has been observed previously in common carp, although the frequency normally does not exceed 1 - 1.5 % (Komen et al., 1991; Cherfas et al., 1995). The origin of this (heritable) phenomenon is thought to be located at initial stages of meiosis (premeiotic endoreduplication or suppression of the first meiotic division) or by suppression of the second meiotic division (Cherfas et al., 1995). It could be responsible for the occurrence of heterozygous individuals within homozygous offspring. Contamination of homozygous gynogenetic offsprings with heterozygous fish has been reported in rainbow trout (Quillet et al.,

Table 2: Controls performed during androgenetic and gynogenetic reproductions presented in this thesis. The irradiation control, in combination with a genetic marker is performed to demonstrate absence of male resp. female inheritance in gynogenesis rep. androgenesis. Segregation patterns of Mendelian markers can be used to demonstrate the homozygous nature of the offspring.

Experiment (chapter)	Irradiation control diploids	genetic marker	Mendelian markers (homozygosity control)	additional control
androgenesis (3)	2.7-5.5 %	coloration: biparentals	none	none
androgenesis (5)	0.0-1.5 %	coloration: biparentals	none	skin grafting: all allografts between isogenic groups accepted (data not shown)
androgenesis (4)	0	none	scalation: in androgenetic offsprings 50 % scaled; in controls 75 %	crosses with androgenetic males yield 100 % male offspring
gynogenesis (6)	0	scalation	none	none
gynogenesis (7)	female 85; not counted	scalation: spontaneous diploids	scalation: in family 69 50 % mirror and 50 % nude offspring	Microsatellites
gynogenesis (8)	4 out of 9 homo-zygous females; not counted	scalation: spontaneous diploids	none	DNA-fingerprints

1994; Scheerer et al., 1991 and Purdom et al., 1985) and in common carp (Cherfas et al., 1995). DNA-fingerprints revealed that in 5 out of 9 gynogenetic inbred strains genetic variation was present (table 3, figure 1). To confirm these findings, homozygosity of the females used to produce the inbred strains was checked using microsatellites (Crooijmans et al., 1996). Seven females were found to be heterozygous. The source of this genetic variation is not clear. The control groups indicate successful induction of homozygosity in family 69 (see table 2). When this family would have merely resulted from spontaneous diploidization due to aberrant processes during meiosis I, only few individuals without any scales (nudes) would have been found since it is known that the recombination rate for this trait is high (0.67: Komen, unpublished). As mentioned previously, we found 50 % nude individuals. Still, 4 out of 6 females from family 69 tested by microsatellites were shown to be heterozygous.

Young et al. (1996) also reported residual heterozygosity in a presumtive homozygous gynogenetic female rainbow trout. Contamination of androgenetic offsprings with heterozygous individuals is not likely (Scheerer et al., 1991; Young et al., 1996), provided that genetic markers proof elimination of the female genome. DNA-fingerprints from rainbow trout offsprings, obtained from homozygous androgenetic parents displayed similar banding patterns (Young et al., 1996). At this moment, we have tested 10 androgenetic genotypes for homozygosity with four microsatellite markers. These preliminary tests suggest eight individuals to be homozygous.

Female 69-26 yielded large amounts of spontaneous diploids in the sperm irradiation control (table 3). This female was used as parent to produce F1 hybrids (chapter 8). In normal fertilizations, spontaneous diploidization processes would give rise to triploid offspring. However, no triploids were found among 70 descendants from this female (data not shown). In the other hybrids, incidence of triploids was low (in total 8 among 150 individuals).

At this moment, only strains 69-13 and 69-45 are considered fully homozygous and genetically identical. The source of residual variation in the other strains remains unclear. For practical purposes, we consider the other strains to have resulted from 2 generations of meiotic-(2pb)-gynogenetic inbreeding and F is estimated 0.96 (0.79 + (0.21 x 0.79); Nace et al., 1970; Bongers et al., chapter 6). The level of isogenicity of the 12 F1-hybrids in chapter 8 is approx. 0.90 ((1 + 0.79)/2).

Table 3: Determination of genetic quality by DNA-fingerprinting and seven polymorphic microsatellite markers of several gynogenetic inbred strains. Occurrence of spontaneous diploids and scaled individuals (genetic marker) in the irradiation control is also presented. For DNA-fingerprints, 5 individuals per strain were tested. Strains were produced from 2 gynogenetic families, initially produced from two hybrid females (codes 69 and 85, see chapter 7)). When producing these families, spontaneous diploids were absent in the sperm irradiation control of female 69 but present in the control of female 85. (?) indicates that the number of scorable fragments on fingerprints was low.

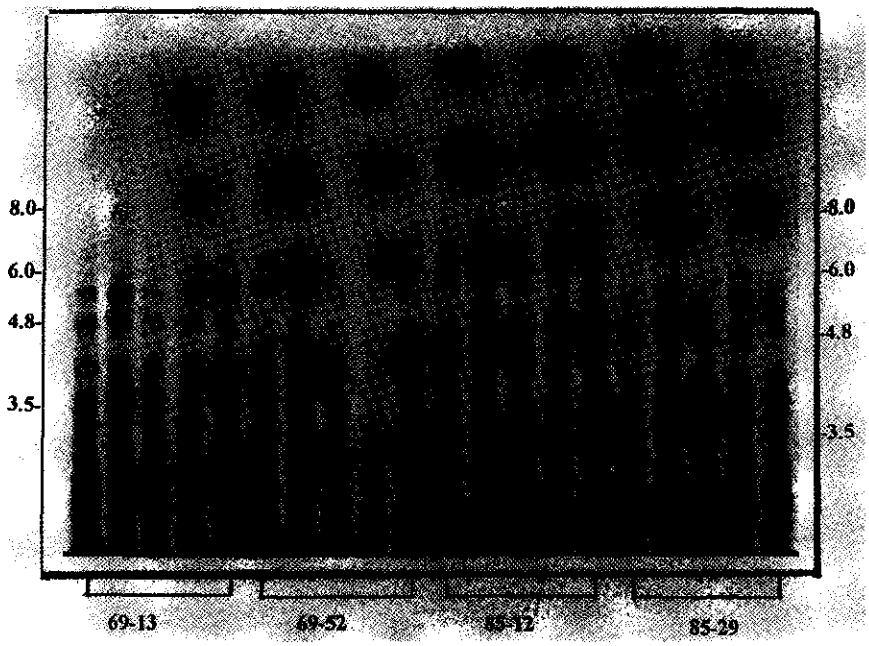
Strain	Spontaneous diploids	Scaled individuals	DNA-Fingerprints	Microsatellites
85-12	yes	no	genetic variation	heterozygous
85-29	yes	no	genetic variation	heterozygous
85-59	no	no	isogenic (?)	heterozygous
69-13	no	no	isogenic	homozygous
69-26	yes	no	genetic variation	heterozygous
69-45	no	no	isogenic (?)	homozygous
69-52	no	no	genetic variation	heterozygous
69-61	yes	no	isogenic (?)	heterozygous
69-82	no	no	genetic variation	heterozygous

4. Conclusions

Genetically uniform strains of common carp have been produced. To produce homozygous individuals, large numbers of eggs have to be treated since (1) high mortality is induced by the heat shock and (2) inbreeding affects vitality and fertility of homozygous animals. The advantages of using genetically uniform carp strains in research is demonstrated in genetic studies and in bioassays. A quantitative genetic framework was developed to enable estimation of genetic variance in homozygous populations. With the model-parameters egg quality and age at maturation, we show that gynogenesis and androgenesis can be useful additional tools in selection programs. In a bioassay to determine toxicity of cadmium, we show that the use of genetically defined strains increases repeatability and reproducability of experiments.

DNA-fingerprints and microsatellite analysis suggested the presence of residual genetic variation in seven out of nine gynogenetic inbred strains. The source of this variation is unclear since all controls during the production of the strains suggested elimination of the male genome and succesful induction of homozygosity in the parents. These findings however stress the importance of monitoring the genetic purity of inbred strains.

Figure 1: DNA-fingerprint of 5 individuals from gynogenetic inbred strains 69-13, 69-52, 85-12 and 85-29. Only in strain 69-13, all individuals showed similar banding patterns. Sizes of molecular weight standards (y-axis) are given in kilobases; their positions have been derived from the locations of bands on ethidium bromide-stained gels photographed prior to probe hybridization



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SUMMARY

Although fishes are widely used in numerous fields of basic and applied research, no demands regarding for example standardization are made thusfar on laboratory fish. In contrast to the situation in laboratory mammals, there are few commercial suppliers and frequently fish with an unknown genetic background and raising history are used.

Inbred strains can be produced by full sib mating. However, approximately 20 generations are needed to obtain (near) homozygous animals. With a generation interval of 1 - 1½ years of female carp, this represents a period of 20 - 30 years. Since (most) fish have external fertilization, manipulation with the sexual products is possible and homozygous individuals can be produced in only one generation. The principle is to eliminate the genetic contribution of one of the parents by irradiation, followed by artificially doubling the haploid genome. Gynogenesis (all female inheritance) is achieved after irradiating the male genome and androgenesis (all male inheritance) after irradiating the female genome. Optimization of gynogenesis and the subsequent development of homozygous gynogenetic inbred strains in common carp, *Cyprinus carpio* has already been performed in an earlier study. In the present thesis, androgenetic reproduction techniques were developed. Inbred strains, congenic strains and F1 hybrids were produced. Common carp was chosen as experimental fish since it is a major species in both aquaculture and research. Genetically uniform strains were tested for their suitability as experimental animal model in genetic research and in bioassays. A detailed analysis of genetic and environmental variation between and within genetically uniform strains was performed.

Disadvantages of using outbred strains are low repeatability between experiments, the possible accumulation of high levels of unexpected inbreeding and the obscuring of treatment effects due to high intrastrain variability. Within genetically uniform strains, the variation is reduced and as a result, less animals (compared to outbreds) are necessary to obtain statistically significant results. We illustrate this advantage of genetically uniform strains with experimental data from a F1 hybrid and partly outbred strain of common carp, both subjected to an antibiotic treatment resulting in reduced gonadal growth (**chapter 2**).

Preliminary results obtained from a single genetically uniform strain should be generalized with the use of a panel of strains. It is shown that the number of strains to be tested depends on the ratio (genetic) variation between strains : (environmental) variation within strains. When selecting a panel of genetically uniform strains, attention has to be paid to genetic relations between strains. It should also be taken into account that in homozygous inbred strains, (genic) dominance and interaction effects are absent, due to the absence of heterozygous genotypes.

When inbred strains are produced the chromosome manipulation treatment induces considerable (environmental) variation. By using F1 hybrids or inbred strains, derived from crossing

genetically identical homozygous individuals this problem can be avoided. Chromosome manipulation should then only be used to generate new homozygous broodstock. Still, variation in F1-hybrids of carp are relatively large as compared to standardized strains of laboratory rodents. It is assumed that their poikilothermic nature make them more susceptible to environmental variation.

To facilitate the production of all-male inbred strains and F1 hybrids, androgenetic reproduction techniques were developed (**chapter 3**). High yields of androgenetic haploids (max. 53.9 %) were obtained using a novel method consisting of stirring eggs in a synthetic ovarian fluid during UV-irradiation (dose: 150-300 mJ/cm^2). Androgenetic origin was checked using a recessive color marker ("blond"). Absence of black fry (dominant) in the offspring confirmed inactivation of the female genome. Subsequent duplication of the male genome was done by heat shocks (40.0 °C, 2 min) . Yields of putative androgenetic diploids at hatching ranged from 7.2 - 18.3 %. Survival of the homozygous larvae 24 days after hatching was high (78 - 89 % of the initial number).
The viability of the YY-genotype in common carp was tested in **chapter 4**. XY-sperm donors for androgenetic reproductions were selected from a congenic strain with stable 50 : 50 sex ratios, produced by repeatedly backcrossing male offspring to a female from a homozygous clone. Sperm donors from a congenic strain were used to minimize the possible influence of minor sex determining genes. Androgenetic offsprings contained 50 % (putative YY) males. The YY-nature of these males was tested after crossing with females from different genetic backgrounds. All-male offsprings were obtained, indicating viability of the YY-genotype in common carp. The remaining 50 % of androgenetic offsprings consisted of females and sterile individuals in equal amounts. This could be due to high levels of crossing over during meiosis I in sex chromosomes of fish. It is suggested that since YY-genotypes are viable and chromosomal interference might be high, sex chromosomes in fish are still evolving.
After androgenetic or gynogenetic reproduction, a large phenotypic variance and high numbers of deformed individuals are generally observed. Large phenotypic variance had previously been reported to be the result of increased genetic variance due to inbreeding and/or increased susceptibility of homozygous genotypes to environmental sources of variation. In **chapter 5** it was tested whether in androgenesis the UV irradiation treatment caused damage to maternal RNA's resulting in aberrant embryonic development, i.e. that UV irradiation could act as a source of environmental variation. Three females and three isogenic inbred males were used to produce 9 isogenic androgenetic offsprings. Yields of androgenetic diploids depended mainly on the egg donor. After raising the larvae for a period of 10 weeks, significant differences were observed regarding the frequency of normal and deformed animals, also depending on the egg donor. It

was hypothesized that this residual maternal influence in the performance of androgenetic carp originated from differences in the abundance of maternal RNA's present at ovulation. In **chapter 6**, more possible sources of environmental variance (V_E) were analyzed: (1) "true" V_E (inter-individual variance), (2) V_E, due to developmental instability (DI, intra-individual variance) and (3) V_E originating from embryonic damage (ED) caused by the chromosome manipulation treatment. In a first experiment we compared three isogenic and one partly outbred strain of carp. The isogenic strains varied in degree of homozygosity (coefficient of inbreeding F: 0 to 0.99) and F in the partly outbred strain was 0.375. It was tested whether homozygous genotypes were more susceptible to true V_E and DI. At 14 weeks of age, length, body weight and number of dorsal fin rays were determined. DI was analyzed by measuring fluctuating asymmetry of 5 bilateral symmetric characteristics. We found the strain with the highest F to display the lowest true V_E. DI was equal in all isogenic strains but highest in the partly outbred strain. This indicated that in our laboratory carp strains the coefficient of inbreeding did not affect V_E. In a second experiment, similar observations were performed on gynogenetic and androgenetic offsprings. Male and female parents came from the same isogenic, normally all-female F1 hybrid. However, some genetic females were hormonally masculinized. As a result, homozygous (EM) gynogenetic and androgenetic reproduction of these parents yielded fully inbred (F = 1) offspring with identical genetic variation and were compared. Partly heterozygous gynogenetic offsprings (2pb-method: F = 0.79) were also produced. Normal fertilizations (F = 0.75) served as controls. It was found that the androgenetic groups showed highest levels of DI and variations caused by ED, followed by 2pb- and EM-gynogenetic groups respectively. We conclude that increased variation within gynogenetic or androgenetic offsprings is the result of ED, caused by the chromosome manipulation treatment.

The consequences of androgenetic or gynogenetic reproduction on distribution of genetic variance were analyzed in **chapter 7**. In homozygous populations, genetic variance only consists of additive genetic variance (V_A). Dominance variance (V_D) and interaction variance (V_I) are thought to be absent. After derivation of additive genetic relations, we concluded that when parents are fully outbred, genetic variance within gynogenetic or androgenetic families equals $V_A + V_E$ and between-family variance equals V_A. Like after conventional inbreeding, V_A is doubled as compared to V_A in the (outbred) base population. This theory was used to analyze experimental data on gonad development and fertility in homozygous gynogenetic carp. Five gynogenetic families were produced from full sib, outbred female parents. For the traits Gonado Somatic Index at 13 months and egg quality (% normal larvae), heritability estimates were 0.71 and 0.72, respectively. These heritabilities were estimated using Gibbs sampling. These results suggest that these traits are influenced by additive genetic effects and that selection can be used

successfully to produce early or late maturing homozygous animals with high egg quality. Especially this last trait is important since homozygous females are in general less fertile than outbred females due to inbreeding depression, thereby hampering the production and maintenance of gynogenetic inbred strains. Our findings show that gynogenesis and androgenesis can be useful additional tools in selection programs. It can be used to estimate directly the additive genetic variance of a certain trait and the breeding value of individual parents. Using a gynogenetic or androgenetic family structure, less individuals per family are needed and the sampling variance of the heritability estimate is reduced, compared to those obtained in a regular half sib design.

In plant breeding and, to a lesser extent in breeding of farm animals, crossing inbred lines to produce hybrids for production purposes is practiced. We examined genetics of testis development in 12 all-male F1 hybrid carp strains, produced after crossing four homozygous androgenetic males with three gynogenetic females (**chapter 8**). These parents were selected for fast or slow gonad development. By regular sampling between the age of 100 and 240 days it was found that the onset of spermatogenesis, ranging from 100 - 130 days after hatching was initiated by additive genetic effects. For the parameter Testis Somatic Index (TSI; range: 2.0 - 5.5 % at 240 days) additive genetic effects also accounted for most of the variance observed. A negative correlation was found between onset of spermatogenesis and TSI. These data fit to a great extent with the established genetic model for gonadal development in the platyfish, *Xiphophorus maculatus*. From analysis of within-strain variations, we conclude that F1 hybrids are suitable as experimental animal models.

The results presented in this thesis show that genetically uniform common carp are excellent animal models in fundamental research (**chapter 9**). Their use in applied research (bioassays) is demonstrated with preliminary data obtained in a toxicological assay. LC50 values of cadmium were determined in 2 laboratories for an inbred and an outbred carp strain. The experiment was repeated with the same inbred strain but with a different outbred strain. Repeatability and reproducibility was only high in the inbred strain.

Problems associated with the development of inbred strains are low survival of eggs, due to the treatment to induce diploidy, and residual genetic variation in several gynogenetic inbred strains. The source of this variation is at present unclear. However, it emphasizes the importance of continuously monitoring the genetic purity of inbred strains.

SAMENVATTING

Hoewel vissen veelvuldig gebruikt worden in fundamenteel en toegepast onderzoek, worden er nauwelijks eisen gesteld aan de gebruikte laboratorium vissen. In tegenstelling tot de situatie in laboratorium zoogdieren zijn er nauwelijks commerciële verkooppunten en daarom worden er vaak vissen met een onbekende genetische achtergrond en opkweek geschiedenis gebruikt. Ingeteelde lijnen kunnen geproduceerd worden via het kruisen van verwanten. Echter, 20 generaties zijn nodig om (nagenoeg) homozygote dieren te verkrijgen. Aangezien vrouwelijke karpers na 1 - 1½ jaar geslachtsrijp zijn representeert dit een tijdsperiode van 20 - 30 jaar. Omdat (de meeste) vissen uitwendig de eieren bevruchten is manipulatie van eieren en sperma mogelijk en homozygote individuen kunnen in één generatie geproduceerd worden. Het principe hiervan is de uitschakeling van de genetische bijdrage van één van de ouders door bestraling, gevolgd door kunstmatige verdubbeling van het haploïde genoom. Bij gynogenese (alleen vrouwelijke overerving) wordt het mannelijk genoom bestraald en bij androgenese (alleen mannelijke overerving) het vrouwelijk genoom. Optimalisatie van gynogenese en vervolgens de ontwikkeling van homozygote gynogenetische inteeltlijnen van karper, *Cyprinus carpio* is reeds in een eerdere studie uitgevoerd. In de huidige studie werden androgenetische voortplantingstechnieken ontwikkeld. Inteeltlijnen, congene lijnen en F1 hybriden werden geproduceerd. De karper werd gekozen als onderzoeksdier omdat het zowel in de visteelt als in wetenschappelijk onderzoek een belangrijke plaats inneemt. De genetisch uniforme lijnen werden getest op geschiktheid als experimenteel dier model in genetisch onderzoek en in bioassays. Een gedetailleerde analyse van genetische- en milieu variatie tussen en binnen genetisch uniforme lijnen werd uitgevoerd.

Nadelen van het gebruik van uitgeteelde lijnen zijn o.a. de geringe herhaalbaarheid tussen experimenten, het mogelijke vóórkomen van onvoorziene inteelt en hoge mate van variatie binnen proefgroepen dat behandelingseffecten kan maskeren. Binnen genetisch gestandaardiseerde lijnen is de variatie gereduceerd en als gevolg hiervan zijn minder dieren (in vergelijking met uitgeteelde dieren) nodig om statistisch significante resultaten te verkrijgen. Dit voordeel van gestandaardiseerde lijnen werd geïllustreerd met experimentele data van een F1 hybride en een gedeeltelijk uitgeteelde karperlijn, beiden onderworpen aan een antibioticum behandeling resulterend in een verminderde groei van de gonade (**Hoofdstuk 2**). Voorlopige resultaten verkregen met een enkele gestandaardiseerde lijn moeten vervolgens worden gegeneraliseerd met behulp van meerdere lijnen. Er werd aangetoond dat het aantal te testen lijnen afhangt van de ratio (genetische) variatie tussen lijnen : (milieu) variatie binnen lijnen. Wanneer een aantal gestandaardiseerde lijnen wordt geselecteerd, moet op de genetische relatie tussen de lijnen gelet worden. Tevens moet men er rekening mee houden dat binnen homozygote

inteeltlijnen, (genetische) dominantie en interactie effecten afwezig zijn vanwege afwezigheid van heterozygotie.
De chromosoom manipulatie techniek induceert een aanzienlijke hoeveelheid (milieu) variatie bij de productie van inteeltlijnen. Door gebruik te maken van F1 hybriden of inteeltlijnen, geproduceerd via het kruisen van genetisch identieke homozygote dieren kan dit probleem ondervangen worden. De chromosoom manipulatie techniek zou alleen gebruikt moeten worden om nieuwe homozygote ouderdieren te genereren. Desalniettemin is de variatie in F1 hybriden van karper relatief hoog in vergelijking met gestandaardiseerde zoogdierlijnen. Aangenomen wordt dat het feit dat vissen koudbloedig zijn hen meer gevoelig maakt voor milieu variatie.

Om de productie van 100 % mannelijke inteeltlijnen en F1 hybrides mogelijk te maken werden androgenetische voortplantingstechnieken ontwikkeld (**Hoofdstuk 3**). Een hoge opbrengst aan androgenetisch haploide larven (max. 53.9 %) werd behaald bij gebruikmaking van een nieuwe methode, bestaande uit het roeren van eieren in een synthetische ovarium-vloeistof tijdens de UV bestraling (dosis: 150-300 mJ/cm^2). De androgenetische herkomst werd geverifiëerd door middel van een recessieve kleur merker ("blond"). Afwezigheid van zwarte larven (dominant) in de nakomelingen bevestigde de uitschakeling van het vrouwelijk genoom. Vervolgens werd het manneljk genoom gedupliceerd door middel van een hitteschok (40.0 °C, 2 min.). De opbrengst aan mogelijk androgenetisch diploiden bij uitkomst uit het ei varieerde van 7.2 - 18.3 %. De overleving van de homozygote larven 24 dagen na uitkomst was hoog (78 - 89 % van het initiële aantal).
De levensvatbaarheid van het YY-genotype in karper werd getest in **Hoofdstuk 4**. XY-sperma donoren voor androgenetische voortplantingen werden geselecteerd uit een congene lijn met stabiele 50 : 50 sex ratios, geproduceerd via het herhaald terugkruisen van mannelijke nakomelingen op een vrouwtje uit een homozygote kloon. Sperma donoren uit een congene lijn werden gebruikt om de mogelijke invloed van minor sex determinerende genen te minimaliseren. Androgenetische groepen nakomelingen bestonden voor 50 % uit (mogelijke YY) mannetjes. Het YY-karakter van deze mannetjes werd getest via het kruisen met vrouwtjes van verschillende genetische achtergronden. Alleen mannelijke nakomelingen werden verkregen hetgeen levensvatbaarheid van het YY-genotype aantoont. De overige 50 % van de androgenetische groepen nakomelingen bestond uit vrouwtjes en steriele individuen, in gelijke hoeveelheden. Dit zou veroorzaakt kunnen worden door een hoge mate van chromosoom-interferentie in sex chromosomen van vissen. Er wordt gesuggereerd dat, omdat YY-genotypes levensvatbaar zijn en chromosoom-interferentie in hoge mate voorkomt, sex chromosomen van vissen nog steeds evolueren.

Na androgenetisch of gynogenetisch voortplanten wordt normaliter een hoge mate van phenotypische variatie en grote aantallen misvormde individuen waargenomen. Hoge mate van phenotypische variatie werd in eerdere publicaties verondersteld het gevolg te zijn van een toename in genetische variatie als gevolg van inteelt en/of een grote gevoeligheid van homozygote genotypes voor milieu variatie. In **Hoofstuk 5** werd getest of bij androgenese de UV bestraling schade aan maternale RNA's veroorzaakt met een verstoorde embryonale ontwikkeling als gevolg, m.a.w. of de UV bestraling als bron van milieu variatie kon fungeren. Drie vrouwtjes en drie isogene ingeteelde mannetjes werden gebruikt om negen isogene groepen androgenetische nakomelingen te produceren. De opbrengst aan androgenetische larven was voor een groot deel afhankelijk van de eidonor. Na een opkweek-periode van 10 weken werden significante verschillen gevonden voor wat betreft de frequenties van normale en misvormde individuen, wederom afhankelijk van de eidonor. Er werd verondersteld dat deze residuele maternale invloed op de kwaliteit van androgenetische karpers veroorzaakt werd door verschillen in hoeveelheid maternale RNA's, aanwezig tijdens ovulatie. In **Hoofdstuk 6** werden meer mogelijke bronnen van milieu variatie (V_E) geanalyseerd: (1) "echte" V_E (variatie tussen individuen), (2) V_E veroorzaakt door instabiliteit tijdens de ontwikkeling (IO; variatie binnen individuen) en (3) V_E als gevolg van embryo-schade (ES) veroorzaakt door de chromosoom manipulatie behandeling. In een eerste experiment werden drie isogene en één gedeeltelijk uitgeteelde lijn vergeleken. De isogene lijnen variëerden in mate van homozygotie (inteeltcoefficiënt F: 0 tot 0.99) en F in de gedeeltelijk uitgeteelde lijn was 0.375. Er werd getest of homozygote genotypes meer gevoelig waren voor echte V_E en IO. Op een leeftijd van 14 weken werden lengte, gewicht en het aantal dorsale vinstralen gemeten. IO werd geanalyseerd via het meten van vijf bilateraal symmetrische kenmerken. Er werd gevonden dat de lijn met de hoogste F de laagste echte V_E vertoonde. IO was gelijk in alle isogene lijnen maar het hoogst in de gedeeltelijk uitgeteelde lijn. Dit duidde er op dat in onze laboratorium karperlijnen de mate van inteelt niet van invloed was op echte V_E. In een tweede experiment werden dezelfde waarnemingen verricht aan gynogenetische en androgenetische groepen nakomelingen. Mannelijke en vrouwelijke ouders waren afkomstig van dezelfde isogene, normaliter 100 % vrouwelijke F1 hybride (F = 0.5). Echter, van sommige (genetische) vrouwtjes werd het geslacht hormonaal veranderd in mannelijke richting. Homozygoot (EM) gynogenetisch en androgenetisch voortplanten leverde aldus volledig ingeteelde (F = 1) groepen nakomelingen met identieke genetische variatie en deze groepen werden vergeleken. Gedeeltelijk heterozygote gynogenetische nakomelingen (2pb-methode: F = 0.79) werden ook geproduceerd. Nakomelingen van normale bevruchtingen (F = 0.75) werden gebruikt als controle. De androgenetische groepen vertoonden de hoogste mate van IO en variatie als gevolg van ES,

gevolgd door resp. de 2pb- en EM gynogenetische groepen. Er werd geconcludeerd dat een toename van variatie in gynogenetische en androgenetische groepen nakomelingen het gevolg is van ES, veroorzaakt door de chromosoom manipulatie behandeling.

De consequenties van androgenetisch of gynogenetisch voortplanten op de verdeling van genetische variatie werd geanalyseerd in **Hoofdstuk** 7. Genetische variatie in homozygote populaties bestaat alléén uit additief genetische variatie (V_A). Dominantie variatie (V_D) en interactie variatie (V_I) worden verondersteld afwezig te zijn. Na afleiding van additief genetische relaties werd geconcludeerd dat, indien ouders volledig uitgeteeld zijn, de genetische variatie binnen gynogenetische of androgenetische families gelijk is aan $V_A + V_E$, en variatie tussen families gelijk aan V_A. Net als na conventioneel intelen is de totale V_A dus verdubbeld in vergelijking tot V_A in de uitgeteelde (basis) populatie. Deze theorie werd gebruikt om experimentele gegevens betreffende gonade ontwikkeling en fertiliteit van homozygote gynogenetische karpers te analyseren. Vijf gynogenetische families werden geproduceerd van uitgeteelde vrouwelijk dieren (volle zusters). De erfelijkheidsgraad van de kenmerken Gonado Somatische Index op 13 maanden en eikwaliteit (% normale larven) werden geschat op resp. 0.71 en 0.72. Deze schattingen werden gedaan met behulp van "Gibbs Sampling". De resultaten suggereren dat deze kenmerken beïnvloed worden door additief genetische effecten en dat selectie met succes kan worden toegepast om vroeg en laat maturerende dieren met goede eikwaliteit te produceren. Dit laatste kenmerk is met name belangrijk omdat, vanwege inteeltdepressie, homozygote vrouwtjes meestal minder fertiel zijn dan uitgeteelde vrouwtjes. Dit bemoeilijkt de productie en het instandhouden van gynogenetische inteeltlijnen. Deze bevindingen illustreren dat gynogenese en androgenese nuttige additionele hulpmiddelen kunnen zijn in selectie programma's. Additief genetische variatie en de fokwaarde van individuele ouders kunnen direct geschat worden. Minder dieren per familie zijn nodig indien een gynogenetische of androgenetische familie structuur gebruikt wordt. Tevens is de variatie van de schatting van de erfelijkheidsgraad kleiner in vergelijking tot schattingen verkregen via een "half sib" familie structuur.

In de plantenteelt en, in mindere mate, in de teelt van landbouwhuisdieren worden kruisingen van ingeteelde lijnen uitgevoerd om F1 hybriden voor productie-doeleinden te verkrijgen. De genetische achtergrond van testis ontwikkeling werd onderzocht in 12 100% mannelijke F1 hybriden, geproduceerd na kruising van vier homozygote androgenetische mannetjes met drie gynogenetische vrouwtjes (**Hoofdstuk 8**). Deze ouders waren geselecteerd voor snelle of langzame gonade ontwikkeling. Door middel van regelmatige monsternames tussen een leeftijd van 100 en 240 dagen werd gevonden dat de start van de spermatogenese (variërend van 100 - 130 dagen) beïnvloed werd door additief genetische effecten. Additief genetische effecten waren ook verantwoordelijk voor het grootste deel van de variatie in Testis Somatische Index (TSI, 2.0

- 5.5 % op 240 dagen). Een negatieve correlatie werd gevonden voor de start van de spermatogenese en de uiteindelijke TSI. Deze resultaten vertonen grote overeenkomst met het geaccepteerde genetische model voor gonade ontwikkeling in het zwaarddragertje, *Xiphophorus maculatus*. Uit de analyse van binnen-lijn variaties werd geconcludeerd dat F1 hybriden zeer geschikt zijn als onderzoeksmodel.

De genetische studies in dit proefschrift tonen aan dat genetisch gestandaardiseerde karperlijnen zeer geschikt zijn als diermodel in fundamenteel onderzoek (**Hoofdstuk 9**). Geschiktheid binnen toegepaste wetenschappen (bioassays) werd aangetoond met voorlopige resultaten verkregen in toxicologische testen. De LC50 waarde van cadmium in een ingeteelde en een uitgeteelde karperlijn werd bepaald in twee laboratoria. Het experiment werd herhaald met dezelfde ingeteelde-, maar een andere uitgeteelde lijn. De herhaalbaarheid en reproduceerbaarheid was alleen hoog voor de ingeteelde lijn.
Problemen, verbonden aan de ontwikkeling van ingeteelde karperlijnen zijn de lage overleving van eieren als gevolg van de behandeling om diploïdie te induceren en residuele genetische variatie in verschillende gynogenetische inteeltlijnen. De oorzaak van deze residuele variatie is vooralsnog niet duidelijk. Het benadrukt echter het belang van het voortdurend controleren van de zuiverheid van inteeltlijnen.

Résumé

(Traduction: Sabine Desmares)

Bien que les poissons soient largement utilisés dans de nombreux domaines de la recherche fondamentale et appliquée, il n'y a jusqu'alors aucune demande concernant les poissons de laboratoire. Contrairement aux mammifères de laboratoire, il y a peu de fournisseurs, et fréquemment des poissons dont on ne connaît ni les descendances génétiques ni le mode d'élevage sont utilisés.

Les lignées consanguines peuvent être produites par accouplements consécutifs entre plein-frères et soeurs. Cependant environ 20 générations sont nécessaires pour obtenir des animaux (presque) homozygotes. Avec un intervalle de 1 an à 1 an et demi entre les générations de carpes femelles, ceci représente une période de 20 à 30 ans. Du fait que la plupart des poissons ont une fécondation externe, il est possible de manipuler les substances sexuelles et des individus homozygotes peuvent être produits en une génération. Le principe est d'éliminer le patrimoine génétique de l'un des parents par irradiation, suivie d'une duplication artificielle du génome haploïde. La gynogénèse (héritage unique de la femelle) est obtenue par irradiation du génome mâle et l'androgénèse (héritage unique du mâle) par irradiation du génome femelle. L'optimisation de la gynogénèse et le développement de lignées consanguines gynogénétiques homozygotes chez la carpe commune, *Cyprinus carpio*, ont déjà été étudiés dans un précédent ouvrage. Dans le document suivant, les techniques de reproduction androgénétique ont été étudiées. Ont été produits, des lignées consanguines, lignées congéniques et des hybrides F1. La carpe commune a été choisie comme poisson expérimental de part son importance en aquaculture et en recherche. La conformité de ces lignées génétiquement uniformes en tant que modèle expérimental animal a été testée en recherche génétique et dans des essais biologiques. Les variations génétiques et environnementales entre et au sein de ces lignées génétiquement uniformes ont été analysées.

Les désavantages à utiliser des lignées exogames sont les faibles répétitions entre les expériences, l'accumulation possible d'un taux élevé de croisements inattendus et la dissimulation des effets du traitement dus à la variabilité au sein des lignées. Dans des lignées génétiquement uniformes, les variations sont minimes et par conséquent, moins d'animaux (en comparaison avec des lignées exogames) sont nécessaires pour obtenir des résultats statistiquement signifiants. Les avantages de ces lignées génétiquement uniformes sont décrits à l'aide de données expérimentales sur un hybride F1 et une lignée partiellement exogame de la carpe commune, tous deux soumis à un traitement antibiotique entraînant une croissance réduite des gonades **(chapitre 2)**.

Les résultats préliminaires obtenus sur une seule lignée (génétiquement uniforme) devraient être généralisés à l'aide d'un ensemble de lignées. Il est démontré que le nombre de lignées à

tester dépend du rapport des variations (génétiques) entre les lignées : variations (environnementales) entre lignées. En sélectionnant un ensemble de lignées génétiquement uniformes, les relations génétiques entre celles-ci doivent être prises en compte. Il est à noter que au sein de lignées consanguines homozygotes, il n'y a ni dominance des gênes ni effets interactifs du fait de l'absence de génotypes hétérozygotes.
Quand des lignées consanguines sont produites, la manipulation des chromosomes induit de fortes variations environnementales. En utilisant des hybrides F1 ou des lignées consanguines issues du croisement d'individus homozygotes génétiquement identiques, ce problème peut être évité. La manipulation des chromosomes ne devrait donc être pratiquée que pour générer un nouveau stock homozygote de géniteurs. Cependant les variations dans les hybrides F1 de carpe sont relativement fortes comparées aux lignées génétiquement uniformes de rongeurs de laboratoire. Il est supposé que leur nature poïkilotherme les rend plus susceptibles aux variations environnementales.

Afin de faciliter la production de lignées consanguines mâles et d'hybrides F1, des techniques de reproduction androgénétique ont été développées (**chapitre 3**). Des rendements élevés d'haploïdes androgénétiques ont été obtenus (max. 53,9 %) en utilisant une nouvelle méthode qui consistait à mélanger les oeufs dans un fluide ovarien synthétique pendant l'irradiation aux U.V. (dose: 150-300 mJ/cm²). L'origine androgénétique a pu être contrôlée grâce à un marqueur de couleur récessif ("blond"). L'absence d'alevins noirs (gêne dominant) dans la descendance a confirmé l'inactivation du génome femelle. La duplication du génome mâle a ensuite été provoquée par choc thermique (40,0 °C, 2 min.). Le pourcentage d'individus supposés diploïdes androgénétiques à l'éclosion était compris entre 7,2 et 18,3 %. La survie des larves homozygotes 24 jours après l'éclosion était élevée (78 à 89 % par rapport au nombre initial).
La viabilité des génotypes YY chez la carpe commune a été étudiée dans le **chapitre 4**. Les donneurs de sperme XY pour les reproductions androgénétiques ont été sélectionnés d'une lignée congénique ayant un sex-ratio de 50:50 produite par croisements en retour répétés d'une descendance mâle et d'une femelle issue d'un clone homozygote. Les donneurs de sperme d'une lignée congénique ont été utilisés pour minimiser l'influence possible de gênes mineurs pouvant déterminer le sexe. Les descendants androgénétiques contenaient 50 % de mâles (supposés YY). Le caractère YY de ces mâles a été testé par croisement avec des femelles d'origines génétiques différentes. Tous les descendants étaient mâles, prouvant ainsi la viabilité du génotype YY chez la carpe commune. Les 50 % de descendants androgénétiques restants comprenaient des femelles et des individus stériles en quantités

égales. Ceci a pu provenir des niveaux élevés d'interférences entre les chromosomes pour les chromosomes sexuels des poissons. On suppose que du fait de la viabilité des génotypes YY et des fortes interférences entre les chromosomes, les chromosomes sexuels chez les poissons sont en pleine évolution.

Après une reproduction androgénétique ou gynogénétique, on observe couramment une variance importante entre les phénotypes et un nombre élevé d'individus difformes. Cette variance entre les phénotypes a été décrite comme étant due à la consanguinité et/ou à la sensibilité plus importante des génotypes homozygotes aux variations environnementales. Dans le **chapitre 5** nous avons étudié si, en androgénèse, l'irradiation aux U.V. pouvait causer des dommages à l'ARN maternel, ce qui résulterait en un développement embryonnaire aberrant, c'est-à-dire que l'irradiation aux U.V. se comporterait comme une source de variation environnementale. Trois femelles et trois mâles consanguins isogéniques ont été utilisés pour produire neuf descendances androgénétiques isogéniques. Les rendements en diploïdes androgénétiques dépendaient essentiellement du donneur d'oeufs. Après une période de 10 semaines, des différences significatives entre les larves ont pu être observées concernant la fréquence d'animaux normaux et difformes qui dépend également du donneur d'oeufs. L'hypothèse serait que cette influence maternelle restante dans les performances des carpes androgénétiques viendrait des différences de quantité en ARN maternel à l'ovulation.

Dans le **chapitre 6**, d'autres sources possibles de la variance d'environnement (V_E) ont été analysées: (1) "vraie" V_E (variance "inter-individus"), (2) V_E, due aux instabilités dans le développement (DI, variance "intra-individu") et (3) V_E provenant de dommages embryonnaires (ED) causés par la manipulation des chromosomes. Dans une première expérience, nous avons comparé trois lignées de carpe isogéniques et une partiellement exogame. Les lignées isogéniques variaient a un certain degré en homozygosité (coefficient de consanguinité F: entre 0 et 0,99) et F, pour la lignée partiellement exogame, était de 0,375. Nous avons testé si les génotypes homozygotes étaient plus sensibles à la "vraie" V_E et à DI. A l'âge de 14 semaines, nous avons déterminé la longueur, le poids et le nombre de rayons de la nageoire dorsale. DI a été analysée en mesurant les fluctuations asymétriques de 5 caractères symétriques bilatéraux. Nous avons découvert que la lignée avec F le plus élevé avait la "vraie" V_E la plus faible. DI était équivalente dans toutes les lignées isogéniques et plus élevée dans la lignée partiellement exogame. Ceci indiquait que, dans nos lignées de carpe de laboratoire, le coefficient de consanguinité n'avait pas d'effet sur V_E. Dans une seconde expérience, des résultats similaires ont pu être observés dans les descendances gynogénétiques et androgénétiques. Les parents mâles et femelles provenaient des mêmes hybrides F1 isogéniques, normalement tous femelles. Cependant quelques femelles

génétiques s'étaient transformées en mâles par un traitement hormonal. Par conséquent, la reproduction gynogénétique et androgénétique homozygote (EM) de ces parents conduisaient à des descendants entièrement consanguins (F = 1) avec des variations génétiques identiques. Ils ont été comparés. Les descendants gynogénétiques partiellement hétérozygotes (méthode 2pb: F = 0,79; 2pb: second globule polaire) ont été également produits. Les fécondations normales (F = 0,75) servaient de contrôle. Il a été découvert que les groupes androgénétiques avaient des DI et des variations causées par ED plus élevées, suivis respectivement par les groupes gynogénétiques 2pb et EM. Nous avons conclu que les variations croissantes au sein des descendants gynogénétiques et androgénétiques étaient dues à ED, causés par la manipulation des chromosomes.

Les conséquences de la reproduction androgénétique et gynogénétique sur la distribution de la variance génotypique ont été étudiées dans le **chapitre** 7. Dans les populations homozygotes, la variance génotypique ne comprend que la variance génétique additive (V_A). La variance de dominance (V_D) et la variance d'interaction (V_I) sont supposées absentes. Après dérivation des correlations génétiques additives, nous avons conclu que, lorsque les parents étaient totalement exogames, la variance génotypique au sein des familles gynogénétiques et androgénétiques était égale à $V_A + V_E$ et la variance entre familles à V_A. Comme après des croisements consanguins classiques, V_A était le double de V_A de la population (exogame) de base. Cette théorie a été utilisée pour analyser les données expérimentales sur le développement des gonades et la fécondité des carpes gynogénétiques homozygotes. Cinq familles gynogénétiques ont été produites avec des parents exogames (plein-soeurs). Concernant les 2 caractères, l'Index Somatique des Gonades à 13 mois et la qualité des oeufs (% de larves normales), l'héritabilité a été estimée respectivement à 0,71 et 0,72. Ces héritabilités ont été estimées suivant l'échantillonnage de Gibbs. Ces résultats suggèrent que ces caractères sont influencés par les effets additifs des gênes et cette sélection peut être utilisée avec succès pour produire des animaux homozygotes à maturation précoce ou tardive avec des oeufs de grande qualité. Ce dernier caractère est particulièrement important puisque les femelles homozygotes sont en général moins fécondes que les femelles exogames, (à cause de la dépression de consanguinité), en gênant la production et maintenance des lignées consanguines gynogénétiques. Nos découvertes montrent que la gynogénèse et androgénèse peuvent être des outils supplémentaires utiles dans les programmes de sélection. Ceci peut être utilisé directement pour estimer la variance génétique additive d'un certain caractère et la valeur d'élevage des parents individuels. En utilisant des familles gynogénétiques et androgénétiques, moins d'individus sont nécessaires

par famille et la variance d'échantillonnage de l'estimation de l'héritabilité est réduite en comparaison avec celles obtenues dans un dispositif traditionel de demi-germain.

Dans la culture des plantes et, dans une moindre mesure, dans l'élevage des animaux, croiser des lignées consanguines pour produire des hybrides est pratiqué dans un but productif. Nous avons étudié la base génétique du développement des testicules dans 12 lignées mâles hybrides F1 de carpes, produites après croisement de quatre mâles androgénétiques homozygotes avec trois femelles gynogénétiques (**chapitre 8**). Ces parents ont été sélectionnés pour le développement, lent ou rapide, de leurs gonades. Par un échantillonnage régulier entre l'âge de 100 et 240 jours, nous avons découvert que le début de la spermatogénèse, entre 100 et 130 jours après éclosion, était déclenchée par des effets additifs des gênes. Pour le paramètre Index Somatique des Testicules (TSI; entre 2,0 et 5,5 % à 240 jours), les effets additifs des gênes expliquent en partie la variance observée. Nous avons trouvé une corrélation négative entre le début de la spermatogenèse et TSI. Des données sont en grande partie en accord avec le modèle génétique établi sur le développement des gonades du platy, *Xiphophorus maculatus*. L'analyse des variations au sein de lignées nous a permis de conclure que les hybrides F1 étaient utiles en tant que modèles animaux expérimentaux.

Nos études génétiques, dans ce document, montrent que les carpes communes génétiquement uniformes représentent d'excellents modèles animaux en recherche fondamentale (**chapitre 9**). Leur utilité en recherche appliquée (essais biologiques) est démontrée à l'aide de données préliminaires obtenues par un test toxicologique. Des valeurs LC50 sur la toxicité du cadmium ont été déterminées dans deux laboratoires pour une lignée de carpes consanguine et une exogame. Cette expérience a été répétée avec la même lignée consanguine mais avec une lignée exogame différente. La répétition et la reproductibilité étaient élevées seulement pour la lignée consanguine.

Les problèmes associés au développement de lignées consanguines sont la faible survie des oeufs, due au traitement qui induit la diploïcie et les variations génétiques résiduelles dans de nombreuses lignées consanguines gynogénétiques. Les causes de ces variations ne sont pas claires aujourd'hui. Cependant, ceci met en évidence l'importance de contrôler en continu la pureté génétique des lignées consanguines.

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Guus

Curriculum vitae

Augustinus Bernard Joannes Bongers werd op 15 september 1963 geboren te Utrecht. Na het behalen van zijn diploma O.V.W.O. aan de r.k. scholengemeenschap "de Klop" te Utrecht startte hij in 1982 met zijn studie Zoötechniek aan de (toenmalige) Landbouwhogeschool in Wageningen. In 1988 rondde hij zijn studie af, met afstudeervakken in de Visteelt en Visserij en Pluimveeteelt. Na het vervullen van de militaire dienstplicht werd hij in januari 1990 aangesteld als onderzoeks-assistent aan de vakgroep Visteelt en Visserij van de (inmiddels) Landbouw Universiteit. Van november 1990 tot en met augustus 1993 was hij werkzaam aan dezelfde vakgroep als toegevoegd onderzoeker. In september 1993 werd een 3-jarige beurs verkregen van de Stichting voor Technische Wetenschappen om het hier beschreven onderzoek te verrichten. Na zijn promotie vertrekt hij als zelfstandig ondernemer naar Frankrijk.