Clones of common carp, *Cyprinus carpio*

New perspectives in fish research

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Stellingen

I Gynogenese is een snelle en effectieve methode voor het produceren van homozygote inteeltlijnen bij vissen.

Dit proefschrift

II De opvatting, dat bij gebruik van U.V. licht voor genetische inactivatie van DNA alleen kleine hoeveelheden sperma succesvol kunnen worden bestraald, is onjuist.

Chourrout, D. 1987. Genetic manipulations in fish: review of methods. In: K.Tiews (ed.) Selection, Hybridisation and Genetic Engineering in Aquaculture. Schr.Bundesforschunsanst.Fisch.Hamburg, 18/19 (Vol I/II). Heenemann, Berlin, Vol. II, pp. 111-127.

III Voor de gynogenetische produktie van homozygote nakomelingen verdienen eieren van niet ingeteelde moederdieren de voorkeur boven eieren van reeds via gynogenese gedeeltelijk ingeteelde vissen.

Dit proefschrift

IV Bij experimenten waarbij geslachtsomkeer bij vissen wordt geinduceerd door toediening van androgenen via het voer, dient de dosering van het hormoon gebaseerd te zijn op het gemiddeld visgewicht in plaats van op de te verwachten gewichtstoename gedurende de behandelingsperiode.

Dit proefschrift

V Bij het verklaren van transplantaat afstoting bij de karper wordt het belang van minor histo-incompatibiliteit tussen donor en ontvanger vaak onderschat.

Dit proefschrift

VI Een mogelijke expressie van H-Y antigeen op mannelijke 8-cellige muize embryo's is niet in overstemming met de veronderstelde rol van dit antigen in de spermatogenese.

Burgoyne, P.S., Levy, E.R. and McLaren, A. 1986. Spermatogenic failure in male mice lacking H-Y antigen. Nature, Vol. 320, pp.170-172.

VII Bij de geslachtsdeterminatie van de karper zijn behalve testis determinerende genen ook ovarium determinerende genen betrokken.

Dit proefschrift

VIII De vergroting van de oogdiameter bij zilveraal, welke een behandeling met gonadotroop hormoon ondergaan, is geen adaptatie aan het diepzee milieu van de veronderstelde paaigronden, maar eerder de expressie van geslachtsrijpheid.

Pankhurst, N.W. 1982. Relation of visual changes to the onset of sexual maturation in the European eel, Anguilla anguilla. J. Fish Biol. 21, 127-140.

IX De constatering dat linkshandige mensen eerder ongelukken maken, vaker last hebben van auto-immuun ziektes en gemiddeld vroeger sterven dan rechtshandigen, werpt nieuw licht op de uitdrukking "twee linkerhanden hebben."

Piet Vroon: de tranen van de krokodil, Ambo (Baarn), 1990

X Risicodragend onderzoek is uitsluitend gebaat bij een onvoorwaardelijke financiering.

XI Het feit dat dieren in prachtkleed door veel mensen als mooi of opvallend worden beoordeeld, impliceert niet dat mensen en dieren vergelijkbare criteria hanteren bij het beoordelen van hun partner.

XII Gynogenese is een koud kunstje

Stellingen behorend bij het proefschrift "Clones of common carp, Cyprinus carpio: new perspectives in fish research" van J.Komen

Wageningen, 5 september 1990

En prononçant le nom du Cyprin que nous allons décrire, on ne rapelle que les contrées privilégiées des zones tempérées, un climat doux, une saison heureuse, un jour pur et serein des rivages fleuris, des rivières paisibles, des lacs enchanteurs, des étangs placés dans les vallées romantiques . . .

Histoire Naturelle de Lacépède
Tome II, poissons, 1876

Contents

1 General introduction 1

2 Introduction to the papers 31

3 Gynogenesis in common carp (*Cyprinus carpio L.*) I: 45
Effects of genetic manipulation of sexual products and incubation conditions of eggs.

4 Gynogenesis in common carp (*Cyprinus carpio L.*) II: 61
The production of homozygous gynogenetic clones and F1 hybrids.

5 Skin grafting in gynogenetic common carp: 83
the development of histocompatible clones.

6 The effects of heterozygous and homozygous gynogenetic inbreeding on sex, 101
gonad development and fertility of common carp (*Cyprinus carpio L.*)

7 The effects of oral administration of 17α methyltestosteron and 123
17β estradiol on gonad development in common carp.

8 Sex control in common carp (*Cyprinus carpio L.*). 141

Summary 155

Samenvatting 160

Chapter 1

GENERAL INTRODUCTION

Summary

In this chapter an overview of various genetic aspects of the common carp is given. First a description of the zoogeografic distribution of wild common carp populations is presented, followed by a section dealing with the history of domestication of these populations in Asia and Europe. A synopsis of some current carp strains, used in this research or frequently mentioned, is given in section 1.3. The inheritance and polymorphism of various marker traits, i.e. scalation, pigmentation, biochemical markers (proteins) and histocompatibility antigens, is described in reference to these strains.

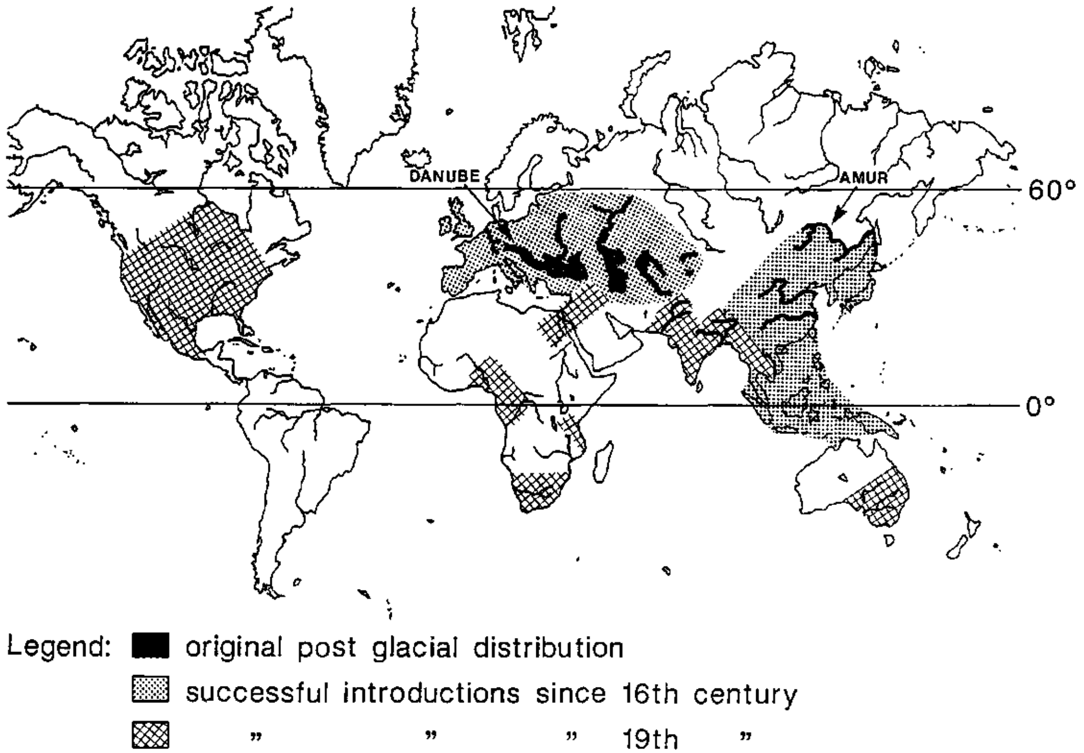
1.1 Zoogeography of the common carp

The common carp, *Cyprinus carpio* (Linnaeus 1758), is perhaps the best known teleost in the world. Today its natural distribution ranges from Western Europe throughout the continent of Eurasia to China, Korea, Japan and South-East Asia and from Siberia south of latitude 60° N to the Mediterranean and India (fig. 1). They have also been introduced in Africa, Australia and North America (Steffens, 1980; Moyle, 1984; Shearer and Mulley, 1978; Wohlfwth, 1984).

Despite this worldwide distribution little is known about the zoogeography of the original wild species. This is partly due to a long history of domestication which resulted in a continuous mixing of escaped or released pond carp with local populations. The existence today of true wild carp is therefore doubtful, since any wild population might include feral carp (Lelek, 1987). The original species *Cyprinus carpio* is generally believed to originate from the great rivers and lakes of pliocene Eurasia, but there are several theories concerning their pleistocene and post-glacial distribution. According to Berg (cit. in Kirpichnikov 1967) carp originally inhabited an unbroken range in Eurasia from the Don and Danube in the West to the Amur drainage basin and China in the Far-East. The presence of carp in the Black Sea during the pliocene is partly substantiated by fossil remnants in lacustrine strata in that area.

During the multiple pleistocene glaciations this domain broke up into an eastern and Western part which today may constitute three subspecies (Kirpichnikov, 1967):

Figure 1 Zoogeographic distribution of common carp, *Cyprinus carpio*



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1- European-Transcaucausian carp: *Cyprinus carpio carpio*

2- Amur-Chinese carp: *Cyprinus carpio haematopterus*

3- South-East Asian carp: *Cyprinus carpio viridiviolaceus*

This distinction is mainly based on differences in morphometric characters i.e. the numbers of dorsal fin rays, lateral line scales, gill rakers on the first gill arch and vertebrae (table 1). Thus, the Amur-Chinese carp have lower counts for dorsal fin rays and gill rakers compared to wild Danube, Don and Wolga carp or carp from various Central-Asian origins. Amur-Chinese carps are also more resistant to low temperatures. South-East Asian carp have been described by Tran Dinh-Trong (1967) in Vietnam and differ from both the Amur-Chinese and European populations in counts of vertebrae, lateral line scales, and gill rakers.

This subspecies separation has been criticized by Balon (1974) who has doubts about the presence of carp in the Danube refuge during the extensive glaciations of the pleistocene. According to Balon, the carp originated in the area of the Caspian/Aral seas from where it spread West to the Danube and East to Asia during post-glacial times. Had carp been present in the Danube during the pleistocene they would certainly have penetrated into Europe and the British Isles during the period of interfluvial connections (Thienemann, 1950). However, many but not all European Cyprinids penetrated Western and Northern Europe after the last (and most extensive) glaciation (Hamilton et al., 1989), and truly endemic species still occur South of the Alps and in the Danube river refuge (Lelek, 1987; Banarescu et. al., 1971). The restriction of carp to the Danube and Don rivers before and after the glaciations might be due to its particular ecological demands such as extensive flooded river banks during springtime and water temperatures well above 15°C (Schäperclaus, 1961; Horvath and Lukowicz, 1982; Lelek, 1987). The river Rhine and Rhône do not fulfill these demands since they obtain their water mainly from melting snow in the Alps.

A more important argument against the existence of two separated populations of common carp as early as the beginning of the pleistocene is the absence of any clear-cut differentiation between the Western and Eastern population. All morphological traits (see table 1) show a gradual change from West to East. The differences in counts for vertebrae between European and Asian common carp disappear when fish are raised at the same temperature (25°C: Moav et al., 1975a; Suzuki and Yamaguchi, 1984) and the different values between populations of carp might thus be the result of the different

ecological conditions, e.g. the higher rearing temperatures. This could particularly be true for the Vietnamese carp. It cannot be excluded that these carp were, as in most South-East asian countries, imported from China by Bhuddist monks as early as the 18th century (Steffens, 1980).

Furthermore crosses between Amur-Chinese and domesticated European carp are fully fertile although gonad abnormalities are noticed in crosses between domesticated European and domesticated Amur-Chinese carp (Hulata et al., 1980). Unfortunately any data on wild Chinese common carp are lacking to sustain arguments for a possible sub-species status of the wild Amur-Chinese population. *Cyprinus carpio* is therefore at present regarded as a single species with locally adapted subpopulations (Balon, 1974).

Table 1 Morphometric counts for common carp from different geographic origins (range of mean values).

Population	Vert.	D.F.	L.L.	G.R.	Ref.
European					
Danube, Don, Volga	36.3-36.8	19.0-19.4	37.6-38.9	23.6-26.5	1
Domesticated	(36-38)	(16-21)	(35-40)	(23-26)	2,3
ibid. at 25°C	34.1-35.3	20.7	36.0-38.0	27.4-28.4	4,5
Central Asia	36.0-36.6	18.3-19.4	36.1-38.9	23.5-26.9	1
South-East Asia					
Amur river	36.6	17.6-18.2	37.8-38.1	20.6-21.3	1
Chinese/Japanese (domesticated)	33.6-34.3	18.6	34.4-34.7	22.4-24.7	4,5
Vietnamese	32.2-34.0	19.4-20.7	31.5-32.9	18.1-19.4	6

Vert = vertebrae; D.F. = soft dorsal fin rays; L.L. = scales in lateral line; G.R. = gill rakers on outer gill arch. References: 1- Kirpichnikov, 1967; 2- Schäperclaus 1961; 3- Steffens, 1980; 4- Moav et al., 1975a; 5- Suzuki and Yamaguchi, 1984; 6- Tran Dinh-Trong, 1967.

1.2 History of domestication

South East Asia The domestication of common carp undoubtedly started in China. Artificial hatching of fish was already in practice in China around 2000 B.C. The development of fish culture grew parallel with the culture of silk worms, as the pupea of the silkworm and their faeces provided supplementary feeding for fish (Lin, cit. in Hickling, 1962). The first treatise on carp culture appeared in 475 B.C. by Fan Li. Carp were captured during the flooding of the rivers, and grown to marketable size in artificial ponds or lagoons. Usually the smaller carp were left in the ponds after seining to spawn and produce new fry the next year. This "broodstock" was supplemented with wild carp from the river. Competition in the ponds was very strong due to the high density, the presence of several fish species and the wide ranges in sizes. Fry had to compete strongly for food and were subject to parasites and infectous diseases. According to Mann (1961), Hickling (1962) and Wohlfarth (1984), this type of pond culture changed little over the centuries. Buddhist monks were probably responsible for some of the early carp introductions in South-East Asia (e.g. Indonesia: Weber and de Beaufort, 1916; Steffens, 1980). In Japan carp culture dates back at least 1900 years. The centres of carp culture were Nagano, Gunma and Yamagata prefectures (Suzuki, 1979).
Europe In Europe carp first appear as "Kuprinos" in the works of Aristotle (Historia animalium). The name might refer to Kypris or Aphrodite, perhaps because of its high fecundity: Aristotle describes how "13 to 14 males chase a female whose eggs they will fertilize" (Hist.anim. VI, 14). Another interesting reference to Aphrodite concerns the word "epitragiai" or hermafrodite. According to Aristotle, "these carp have neither milt nor eggs, are solid and fat, and are considered the best" (Hist.Anim. IV, 11). The passage clearly indicates that carp were eaten at that time. Contrary to common belief however, it is doubtful whether Romans appreciated this fish and kept them in their Piscinae. Balon (1974) reconstructs a possible introduction from the Amber road - Danube crossing to the Roman empire but there are no texts to sustain these presumptions. On the contrary: Aelianus (2nd century A.D., De Natura animalium XIV, 23 and 26) decribes the catch of large numbers of black carp by local inhabitants of the Ister (= Danube). The specific mention of black carp indicates that the Romans knew carp, but were not familiar with Danube (black) carp.

Carp are also notoriously absent in the famous cookbook of Apicius (4th century A.D.),

and in the Deipnosophistae (Athenaeus, 3th century A.D.), a compilation of text-quotations on food presented as a dinertable conversation, carp are only mentioned twice (6 lines in an entire volume dedicated to fish!). Since both Apicius and Athenaeus describe the culinary preferences of the Roman elite, the absence of carp can be taken as circumstantial evidence that they were not a delicacy in ancient Rome. Most assumptions of a Roman appreciation of carp stem from Cassiodorus (Variae XII,4; 6th century A.D.). In his text carp appear as "carpa". The context indicates that carp from the Danube are a "novitatibus", to be part of a King's table (among other things) to show his power and wealth. The use of the local name "carpa" instead of the Latin name Cyprinus (Plinius, Naturalis Historia IX, 58), suggests that carp were not well known.
The first reliable references to carp culture and consumption appear as late as the 12th century A.D. Albertus Magnus (1193-1280) refers to carp-growing in ponds, while Hildegard Von Bingen (1098-1179) from the Bavarian convent of Benedictines elaborates on their preparation. However, it is only with the beginning of the Renaissance in Northern Europe that books solely dedicated to pond culture and carp start to appear (Dubravius: on fish ponds, 1547; Strumienski: O Sprâwie, sypániu, wymiérzániu, i rybieniu, 1573; Taverner: Certain experiments concerning fish and fruit, 1600). It is in this period that carp were transported all over Europe, "packed with snow and with a piece of bread soaked in gin in their mouth" (Bloch, 1789). Around 1514 carp reach England, and in 1560 Holland and Denmark. Bloch also relates of transports by boat from Prussia to Sweden. By the end of the 16th century Danube carp had spread throughout Europe, and Bohemia had become the centre of pond culture (Berka, 1985). Ponds were constructed in Bavaria, Austria, Brandenburg and Prussia where the nobility made a living from selling fish (Shaw, 1804). During the 19th and 20th centuries carp culture in Germany and Bohemia became more sophisticated, due to the development of controlled spawning in separate ponds (Dubisch, 1813-1888), and many carp strains were developed. On the other hand, in most Western-European countries a gradually improved infrastructure resulted in a more constant supply of fresh and cheap seafish. In these countries pond culture became more or less obsolete (Hickling, 1962). However, feral populations of carp continued to play a minor role in fisheries and angling.

1.3 Strains of common carp

The different intensities of selection in locally adapted wild/feral stocks, Chinese extensive pond culture, and European intensive pond culture, had different impacts on the genetic structure of these carp populations. In Europe many distinctive races were developed, while in China cultured carp remained more or less genetically similar to wild carp. Today, the most successful carp strains are almost all crossbreds of different groups of inbred European carp, or crossbreds between inbred European carp and wild/-feral carp or Chinese carp. The strains that are mentioned in this Ph-D study are described in the following sections.

Germany and Bohemia

During the 19th century many distinctive races of common carp had been developed in Germany and Bohemia. They were local strains which were adapted to the local pond conditions and climate, and more or less inbred since most farmers used very few (1-10 !) broodstock animals to produce large numbers of fry. Full-sib matings were therefore inevitable (Schäperclaus, 1961). At the turn of the century an attempt to standardise the major carp races was made (Walter, 1901; Schaperclaus, 1961; Wunder, 1986). The discriminating characters were body shape and scalation:

Aischgrunder carp were developed in Bavaria during the past 300 years. They are mirror carp with very few scales, and are characterised by their high-backed shape, caused by an inheritable deformation in the spinal column (Chondrodystrophy: Wunder, 1949). This race apparently became extinct in 1956 (Steffens, 1980). Today the Aischgrunder shape is still present in some Hungarian and Yugoslavian strains which were produced from Aischgrunder x Galician crosses.

Lausitzer carp are fully scaled stretched carp of a grey colour which were grown in sand bottom ponds under unfavourable climatic conditions.

Bohemian carp are similar to Lausitzer carp but are mainly cultured in mountain areas with vulcanic soils and acidic water. After the second world war most local races of Bohemian carp disappeared. Today all Czechoslowakian strains are called Bohemian. They include both stretched and high-backed carp.

Galician carp are intermediate in shape between Lausitzer and Aischgrunder carp. They were bred by Alfred Gasch (Wunder, 1986) around 1900 and extensively promoted by

Burda (Schaperclaus, 1961). They have a significantly higher growth rate than most other races. As a result Galician carp were crossed with many local strains and eventually replaced all other German races. During the two world wars many carp strains disappeared while the remaining populations became extensively mixed. Post-war carp strains were reconstructed using Galician x Lausitzer carp but the original races had disappeared. The importance of the Galician carp stem from their pre-war export to other countries were they were often involved in the development of new strains.
German mirror carp (Galician x Lausitzer?) from the Harz area were imported by the Wageningen Agricultural University in 1981. They are called "D".

Hungary

In Hungary, carp breeding programmes are based on inbreeding followed by crossbreeding. In 1962 10 different Hungarian local "landraces" (strains) were collected and bred by strict sibmating at the Fisheries Research Institute at Szarvas. Effective hybridization was employed within these strains to improve commercially important characteristics such as survival rate of fry and fat content at harvest (Bakos, 1979). One highly inbred strain, called Tata, shows inbreeding depression and a decreased resistance to infections with *Aeromonas salmonicida* (Sövenyi et al., 1988). Some homozygous scaled individuals of this strain were send to the O.V.B. in the sixties to participate in the 25% hybrid breeding programme (pers.comm. Bungenberg de Jong). Another strain, inbred for 4 generations, arrived via the Fish Culture Experimental Station Golysz (Poland) at the Wageningen Agricultural University in 1986. This strain is called R8.
In 1978 the Hungarian breeding programme was extended with the development of gynogenetically inbred lines from different carp strains. Three strains were used: Nasice (from Yugoslavia), Göd, and Dinnyes (both from Hungary) (Nagy et al., 1984). An extensive growth test involving these gynogenetic inbred strains and their hybrids showed that most genetic variation for growth is non-additive and that the crossbreds display a high degree of heterosis (Nagy, 1987).

The Netherlands

In the Netherlands self-sustaining populations of feral carp are rare. They are believed to be the progeny of fish escaped from ponds in Germany and Holland since the 16th century (Hoek, 1895). In Holland, commercial carp culture never has been an important

industry. In 1899 the first and only commercial carp farm at Valkenswaard started with a mixture of introduced German carp, including Lausitzer, Bohemian and Galician carp (Pennekamp, 1899). The progeny of 50 carp produced on this farm and introduced to Britain in 1934, today still exist as the famous Redmire population (Clifford and Arbery, 1984). In 1952 the management of the farm was taken over by the Organisation for Improvement of Inland Fisheries (O.V.B.). During the following 20 years this organisation developed 3 different strains of carp. Their crossbreds have been stocked in most inland waters to enhance sportfishing (Raat, 1983):

Valkenswaard mirror: in 1956-1957 the original carp stocks at Valkenswaard were culled from leather and line carp (see also 1.4). The remaining fish were bred under stringent mass-selection for growth rate, stretched body shape (no spinal deformation) and disease resistance, to produce the Valkenswaard (VW) mirror carp (Bungenberg de Jong, 1964). Some of the scaled carp from this stock were used to produce a group of homozygous scaled carp. Samples from the Valkenswaard mirror carp strain were send to Israel in 1962 (see Israel).

Hol-B In 1957 a second strain was produced and selected from the offspring of 5 females and 6 males of Aischgrunder x Galician ancestry (courtesy Dr Wunder). Crossbreds between this strain and the VW strain were send to Israel where they became known as Hol-B.

Wild (feral) carp, caught in a Frysian lake near Workum, were used to produce a closed line of "wild" carp. They differ from domesticated carp in body shape, musculature and swimbladder dimensions (Boddeke, 1966). The Wild carp strain is used in the production of 25 % wild hybrids for sportfishing purposes. These 25 % wild hybrids are crossbred progeny of (Wild carp x homozygous scaled carp) x VW mirror carp. For homozygous scaled carps both Valkenswaard and Tata carps are used. Extensive angling experiments have shown that wild carp are more difficult to catch and have better fighting endurance than the domesticated strains. Capture and endurance of the hybrids is intermediate (Beukema, 1969; Raat, 1985). The hybrids have successfully been used in studies on the optimalization of growth in common carp (Huisman, 1974). In 1978 a number of VW mirror carp and 25 % wild hybrids were transferred to the Wageningen Agricultural University were they are registered as "W" carp. The (D x W) F1 broodstock mentioned in this thesis is the progeny of a mirror carp male from the D group (see above) crossed with a scaled female from the W group (25 % wild hybrid).

Israel

In Israel carp farming was initiated in 1939 through a number of introductions, mainly from Europe (Tal and Sheluvki, 1952; Yashouf,1955). Today, commercial breeding of carp is based on strain crossing, exploiting a high degree of heterosis for growth rate (Moav et al., 1975b). During the course of an extensive genetic programme, which started in 1960, a number of useful strains and crossbreds were developed and tested. A short summary of their characteristics is presented (see also figure 4):

Hol-A and Hol-B. These strains were imported from Holland in 1962. The Hol-A strain is equivalent to the Valkenswaard VW mirror carp. Hol-B is the crossbred of this strain with the German strain (see above). A comparison of these strains with local Israeli strains revealed that Hol-A carp (VW) were highly inbred (Moav et al., 1964): their growth rate was the lowest of all tested groups. A high percentage of these carp had skeletal malformations. Hol-A mirror carp progeny were characterised by the possession of many large scales (Streuschupper, see 1.4). Hol-B carp grew much better and were kept as a closed line for further tests.

Blue-grey. This inbred strain was developed from local Israeli carp in 1960 (Wohlfarth et al., 1964), and was found to be highly susceptible to swim bladder inflammation (see 1.4; Hines et al., 1974). The individuals in this strain are marked by homozygosity for two pigment mutations, grey and transparent (see 1.4 and figure 4.2).

Gold. Another inbred strain produced from local Israeli carp in 1963. The carp in this strain are marked by homozygosity for a recessive mutation producing bright orange pigmentation (see 1.4 and figure 4.3).

Nasice. This strain was imported from Yugoslavia in 1970. It maintains many of the characteristics of the Aischgrunder x Galician ancestry, including a high back and skeletal deformations. Their performance under less optimal pond conditions is considerably reduced. They also showed an high incidence of epidermal epithelioma (Hines et al., 1974). Crossbreds of the Blue-grey and Nasice strain are resistant to both diseases.

Dor-70. This strain resulted from a large two-way selection experiment conducted between 1965 and 1970. The details of this experiment are described in Moav and Wohlfarth (1976). The starting population consisted of 5 different familial lines, collected at different fish farms in Israel. During the experiment inbreeding was avoided by using large numbers of carp and by crossing between replicate groups within selected lines.

The results after 5 years suggested that European carp had reached a selection plateau for growth while maintaining a large genetic variance (Moav and Wohlfarth, 1976). The group with the best growth performance was designated Dor-70. This group shows excellent hybrid vigour in crosses with other strains (Gold, Nasice, and Big-belly, see below). In Israel the most succesfully used crossbred is Dor-70 x Nasice (Moav et al., 1975b; Wohlfarth et al., 1975; Wohlfarth et al., 1980). Samples of Dor-70 fish have been sent to Hong Kong, Brazil, Panama, and South-Africa (Wohlfarth et al., 1980; Sin, 1982). Inbreeding of Dor-70 animals produces reduced growth rate and inbreeding depression, manifested by a high incidence of skeletal deformations (Brody et al., 1976). In 1980 2 males and 2 females were transferred from Dor to the Wageningen Agricultural University via the O.V.B. (courtesy Dr. Wohlfarth).

Table 2 Comparison of relative magnitudes of various traits in common carp from European and Chinese origin.

Trait	origin	
	European	Chinese
Growth rate		
-juvenile (up to 3 months)	low	high
-post juvenile (different environments)	high	low
Tolerance to suboptimal environment	low	high
Weight difference between sexes	small	large
Seine escapability	low	high
General viability	low	high
Body shape	high	stretched
Onset of sexual maturity	late	early
Gonado-somatic index	10-15 %	30-40 %

Adapted from Wohlfarth et al., 1975; Moav et al., 1975b; Hulata et al., 1974; 1976; 1982; 1985).

China

As already outlined no specific strains of carp were developed in China. Chinese common carp are collectively termed "Big-Belly" because of their high gonado-somatic index (30 % or more!; see also figure 4.4). They are distributed over most South-East Asian countries (Hickling, 1962; Bardach et al., 1972). Big-belly's from Taiwan and Hong Kong were imported to Israel in 1970, where they were tested in crossbreeding experiments with European strains (Gold, Blue-grey, Dor-70, Nasice, Hol-B). The crossbreds show heterosis under crowded pond conditions with heavy manuring, but occasionally have gonad malformations (Hulata et al., 1980). A comparison for various characteristics between European strains and Big-belly's is presented in table 2.

1.4 Marker traits

Scalation

The different fenotypes for scalation in carp are well known. Based upon the distribution and number of scales, four different types are distinguished (figure 2). Investigations by Probst (1953) and various Russian scientists (in Kirpichnikov, 1987) on the inheritance of these scale patterns showed that they are induced by mutations in two genes, 'S' and 'n'. The genes are responsible for wild type scalation. The dominant mutation N is lethal in homozygous condition. The fenotypes shown in figure 2 have the following characteristics and genotypes:

- scaled carp (fig. 2a.). Wild type. Scales completely cover the body in a regular fashion. Genotype S/S;n/n or S/s;n/n.
- mirror carp (fig. 2b). Carp are irregularly scaled. Scales are enlarged. The degree in which they cover the body varies from almost complete ("streuschupper") to almost absent. Genotype s/s;n/n.
- linear carp (fig. 2c). Carp have a regular row of scales along the lateral line but are otherwise irregularly scaled. Genotype S/S;N/n or S/s;N/n.
- nude or leather carp (fig.2d). In these carp scales are absent or nearly absent. In all cases the line of scales along the entire back from head to tail is interrupted. Genotype s/s;N/n.

The gene S (s) is used in our experiments as a control for sperm irradiation. The males are heterozygous scaled (S/s) while the females are s/s. Mirror carp with few scales were used in skin grafting experiments (see chapter 5).

The mutant N has a pronounced pleiotropic effect (Probst, 1953; Wunder, 1960; Steffens, 1980; Kirpichnikov, 1987). Homozygous N/N larvae die during or shortly after hatching and have a characteristic "comma" shape, comparable to the haploid syndrome (see chapter 3). Heterozygous N/n fish generally have a reduced viability and disease resistance, and often show deformed dorsal, caudal and anal fins. Their growth rate is reduced due to an increased fat metabolism. All these effects are more pronounced in nude carp than in linear carp. Together they suggest that the N/n gene might represent a deletion in a chromosomal region involved in mesodermal differentiation. Because of the negative effects of N, linear and nude carp have been systematically culled from most carp strains (e.g. VW carp, see 1.3).

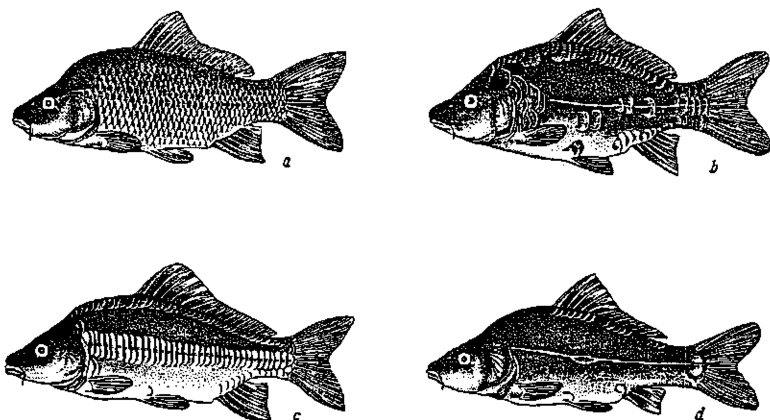


Figure 2 Types of scaling in the common carp

a) scaled (S/S;n/n or S/s;n/n), b) Mirror (s/s;n/n), c) Linear (S/S;N/n or S/s;N/n) and d) Nude (s/s;N/n). from Kirpichnikov, 1987

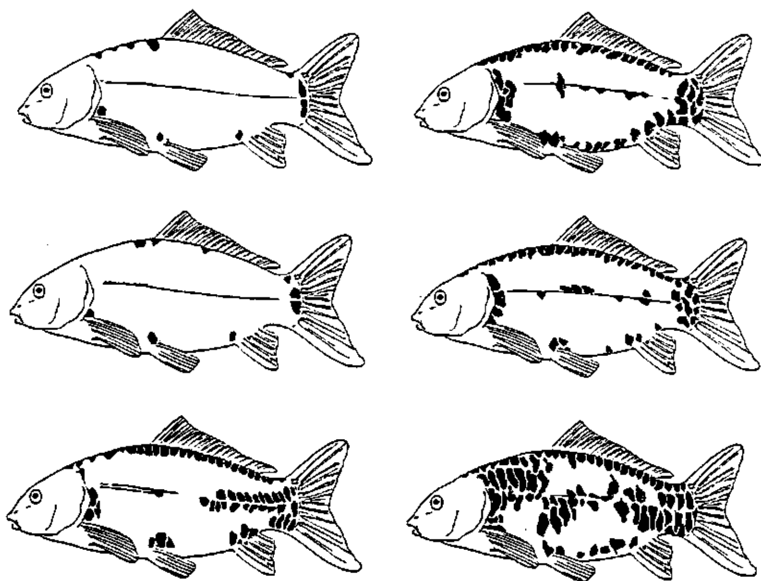


Figure 3 "Fingerprint scalation in clones and F1 hybrids of common carp. Homozygous clones 11 and 22; F1 hybrids 13, 14, 23 and 24.

However, it is well known that even in the absence of N, some carp can have a fenotype that is almost like linear or nude (Schäperclaus, 1961; Wunder, 1986). Hol-B carp often display a linear fenotype (Moav et al., 1964), and in our gynogenetic experiments we discovered nude carp with a mirror carp genotype. Some of these nudes show all the characteristics of true nude carp, including the fin deformations and increased fat metabolism (Komen, unpublished results). The inheritance of this fenotype appears to be monogenic recessive. These observations support the suggestions of Probst (1953) concerning the existence of a multiple allelic system of both "n" and "s" with alleles of different strength. Such a system might explain the high heritability of the variable fenotypes of scalation found in mirror carp (Wunder, 1986). In our experiments we have used the s and n gene complex to produce "fingerprint" fenotypes to mark our clones and F1 hybrids (see figure 3 and chapters 5, 6 and 8).

Pigmentation

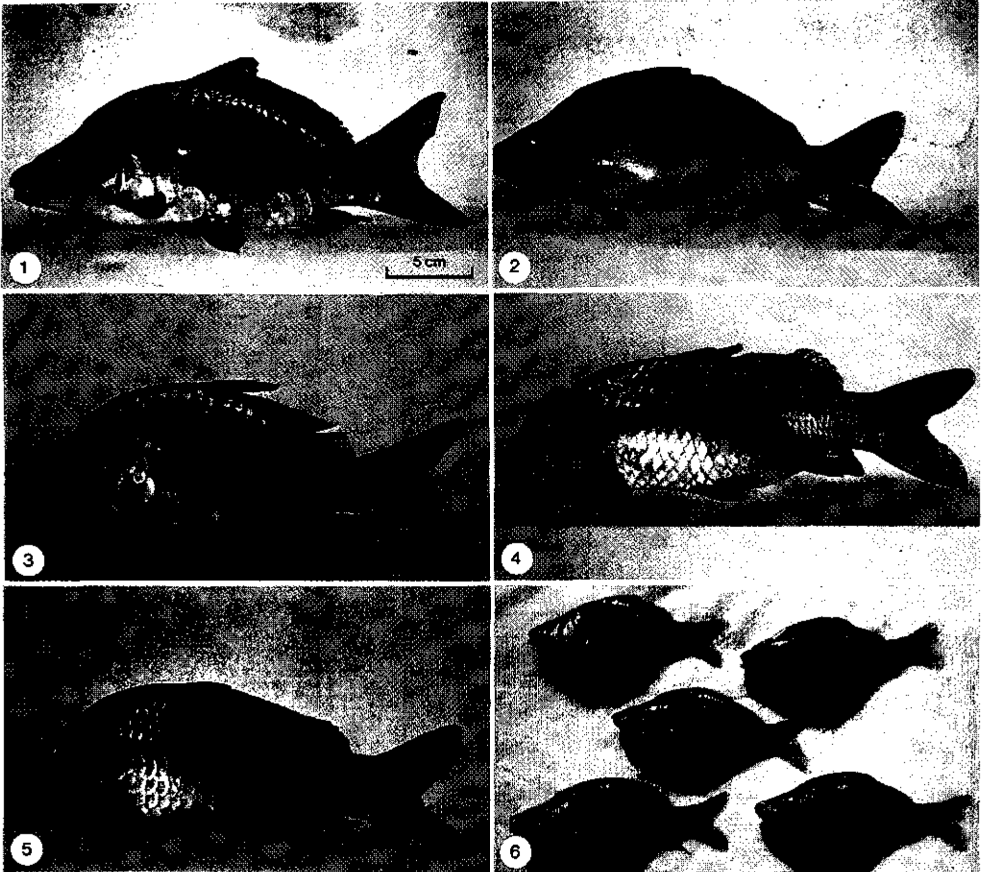
Colour variants are well known in ornamental carp (Koi or Higoi carp), but coloured carp also occur in ordinary domesticated strains, both in Europe (figure 4) and Asia (Buschkiel, 1938; Tran, 1967). Although the inheritance of some of these colour variants has been investigated, it is usually not clear if these studies describe phenotypic variations of the same genotype or different genotypes. Colour is a highly variable trait, subject to environmental influences and overall genotype (genetic background). The interpretation of colour inheritance is therefore difficult and only allelic tests can decide whether colour variants are genetically different or not.

Colour variants in common carp are determined by the relative amount of melanophores, xanthophores and guanophores:

- absence of melanophores result in yellow or orange fish.
- absence of guanophores result in transparent fish (Alampia)
- absence of xanthophores result in grey or "steel" fish
- absence of both melanophores and xanthophores result in white fish

Yellow and orange fish have been analysed by Katasonov (1978) in hybrids of ordinary common carp with Japanese ornamental common carp. Backcrossing of F1 hybrids to parental Japanese carp produce 25 % orange carp. It was concluded that the orange pigmentation resulted from homozygosity for mutations in a duplicated gene B (Black), involved in melanophore formation.

Figure 4 Different types of pigmentation in some strains and crossbreds of common carp.



1) Normal pigmentation (Blue-Grey x Gold).

2) Transparent (Blue-Grey). Note transparent operculum and blueish hue.

3) Gold. No melanophores and uniform orange pigmentation.

4) Normal pigmentation (Big-Belly), note extended abdomen.

5) Gold crossbred (Gold x Big-Belly).

6) Blond fingerlings (Polish inbred strain).

(Photographs 1, 2, 3, 4 and 5 courtesy Dr. G. Hulata; Photograph 6 courtesy Dr. J. Szumiec)

Homozygous b1/b1;b2/b2 fry completely lack melanophores. Mature animals are yellow, orange or even red, with a dark streak along the dorsal/posterior trunk, and with isolated regions of black pimentation on the lateral sides (see figure 4.6). The mutations might affect the migration of melanophores from the neural crest (Shepard, 1961; Lamers et. al., 1981). The residual trunk pigmentation apparently has a different origin - (Shepard, 1961).

In our experiments a similar set of mutants termed "blond" was found (chapter 4). Blond carp were pale yellow, or lemon coloured. Viability of the blond embryos is slightly reduced (Katasonov, 1978; Komen, unpublished results). The red carp described by Nagy et al., (1979) are also homozygous for a duplicated recessive gene, in his experiment termed p and r. They are probably identical to the b1 and b2 genes. The high variability of the yellow/orange colour depends on the relative contents of red and yellow pigment in the xanthophores (Matsumoto et al., 1960), as well as the genetic background: homozygous gynogenetic offspring of a single female produced a wide array of blond fenotypes (Komen, unpublished results).

The "gold" mutant used to mark the Gold inbred line (see figure 4.3) is also bright orange. Its inheritance is monogenic recessive (g) and is thus different from blond. The gene g appears to be dominant in crosses with Big-Belly carp (Hulata, pers. comm.; figure 4.5).

Transparent carp are, in contrast to the afore mentioned colour variants, easily recognized, due to the absence of a reflecting layer in the skin. This condition is termed alampia and results from an underdevelopment or absence of guanophores. In Germany, transparent carp are known as "blaulinge" (Schäperclaus, 1961; Steffens, 1980). The inheritance is unequivocal monogenic recessive. The genes bl_d, bl_p and bl_i, described by resp. Probst (1949), Wlo/dek (1963) and Wohlfarth et al., (1964) are probably all identical. Transparency has been used in our experiments as a marker for gynogenesis (chapter 4). In order to avoid confusion with other gene codes, we have termed this mutant "tp".

Grey carp have been less well studied. According to Katasonov (1978) the inheritance of grey (also termed "steel" and "blueish") is monogenic recessive. The gene is termed R (red) and the mutant r. Grey animals are well known in European carp stocks but a genetic analysis has not been performed (Schäperclaus, 1961; Steffens, 1980). Grey carp have also been noticed in the VW strain (see 1.3; Bungenberg de Jong, 1964).

In Israel grey (= r?) is combined with transparent to mark an inbred line (Blue-Grey, see figure 4.2; Wohlfarth et al., 1964).
White carp are a combination of b1, b2 and r (Katasonov, 1978). White carp have only been described in ornamental carp.
Two dominant mutations, D and L, have also been described only in ornamental carp (Katasonov 1973; 1974, 1975). The gene D produces a pattern of light bands characteristic for ornamental carp. The gene L is lethal in homozygous condition. In heterozygous state it induces a stable contraction of melanophores. Both genes are thought to have a pleiotropic effect on body shape, but these observations need more research.

Biochemical markers

Polymorphic enzymes and other electrophoretically detectable protein variants have had few applications in common carp. The major reason for this is the low level of polymorphism displayed by most proteins tested, except transferrin. Thus, Brody et al.(1979) investigated 33 protein loci in the Nasice, Dor-70 and Big-Belly strains and found only 5-6 polymorphic loci. The Nasice strain was highly inbred and showed only polymorphism for malate dehydrogenase and transferrin. Five polymorphic protein loci out of 29 tested were also found in a comparative study between Italian carp from different geografic origins (Cataudella et al., 1987). All other loci tested, both in Brody's and Cataudella's study, were fixed for the same allele in all populations. The narrow genetic basis of many introduced populations was demonstrated by Shearer and Mulley (1978) for Australian carp: only two loci were polymorphic. A summary of polymorphic enzymes is presented in table 3.
Transferrins are coded for by a single locus with polymorphic alleles. According to Valenta (1976) more than 7 different alleles with decreasing electrophoretic mobility (A to G) can be discerned, while Brody et al., (1979) discern 6 loci (FF, F, I, 2, 1, S).
According to Brody et al., (1979) their S allele might correspond to the G allele of Valenta (1976), but the other alleles are difficult to compare. Despite the large number of alleles few differences are found between most European carp strains. In the Bohemian carp the most frequent alleles are D, E and G, while B is very rare. In the Dor-70 and Nasice strains only S (G?) and F (D?) occur. Chinese Big-belly's are more polymorphic with all six alleles present (Brody et al., 1976; ibid. 1979). The Wageningen

inbred carp strains (including Dor-70 and R8, see 1.3) are all fixed for the same two alleles, D and G. Only the "W" strain (see 1.3) shows polymorphism (Van Muiswinkel et al., 1986; Pourreau, 1990), which is in accordance with its hybrid origin. These observations indicate that transferrin alleles become rapidly fixed in small non-random breeding populations. Their significance in genetic studies involving European carp strains is therefore limited. Why D and G were preferably fixed in the European population is not clear, but a possible relationship with disease resistance has been implicated (Suzumoto et al., 1977). However, preliminary studies on the resistance to *Aeromonas salmonicida* infections in different inbred strains did not show a relationship with the transferrin genotype (Pourreau et al., 1990; Houghton et al., in preparation).

Table 3 Polymorphic enzymes in various tissues of common carp.
(L = liver, B = blood, M = muscle, Br = Brain)

Enzyme	Locus	E.C. nr.	Tissue	Alleles	Ref.
Carbonic anhydrase	Ca-1	4.2.1.1.	L	2	2
Esterase	Est-1	3.1.1.2.	L	2	1
	Est-3		L/B	3	1,2,6
	Est-4		L/B	2	1,2
Lactate dehydrogenase	LDH-B_1	1.1.1.27	Br/L	2	2,3,5
	LDH-C_2		L	2	1,4,5
Malate dehydrogenase	MDH-B	1.1.1.37	M/B	2	1,6
Phosphoglucomutase	PGM	2.7.5.1	M/B	3	1,2

(1- Brody et al.,1979; 2- Cataudella et al., 1987; 3- Engel et al., 1973; 4- Shearer and Mulley, 1978; 5- Valenta et al., 1976; 6- Cherfas and Truveller, 1978).

Histocompatibility antigens

Immunogenetic markers, associated with the major histocompatibility complex (MHC), are extremely useful tools in mammalian population genetics (Klein, 1982). The gene products of the MHC are highly polymorphic cell membrane glycoproteins. At the fuctional level they are separated in class I and class II histocompatibility antigens. Class

I antigens are the classic transplantation antigens which can be identified by acute graft rejection, T-lymphocyte mediated cell lysis, or specific allo-antisera. Class II antigens are involved in cell-cell interactions and can be identified by mixed leucocyte reactions or specific allo-antisera. There is compelling evidence that common carp possesses a MHC analogue. Skin grafts are invariably rejected in an acute fashion, with associated specific memory formation (Hildemann, 1970; Botham et al., 1980). Carp lymphocytes are capable of stimulating and responding in the mixed leucocyte reaction (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984). Specific allo-antisera have been raised by injecting fish with purified leucocytes (Kaastrup et al., 1989). Finally, in the related crucian carp (*Carassius auratus langsdorfii*), adoptive transfer of immunity by pronephric cells is successful in isogenic recipients and some allogeneic recipients with a weak histocompatibility (H) disparity, but not in xenogeneic or strong H-disparate allogeneic recipients (Nakanishi, 1987a; 1987b). The very fact that carp can mount a specific immune response against allogeneic tissue from closely related carp indicate that the glycoproteins involved are polymorphic both in their public (interstrain) and private (inter-individual) specificities. The immunogenetic tests which have been used to discriminate between individuals include:

- allograft exchange.
- mixed leucocyte reaction (one-way and two-way).
- haemagglutination test with specific allo-antisera.

Allograft reactions in common carp are discussed and reviewed in chapter 5. The main conclusions are that cumulative minor histocompatibility differences can result in acute graft rejections which mimic major histocompatibility differences. This phenomenon is well known in mammals and amphibia (Hildemann and Cohen, 1967). In the conventional inbred strains studied (e.g Dor-70 and R8; Boon, pers. comm.) grafts were all rejected in an acute fashion. These results indicate that in an inbred strain minor H loci become fixed for different alleles in different individuals.

Mixed leucocyte reactions (MLR) in carp have been studied by Caspi and Avtalion (1984) and in our laboratory (Gloudemans et al., 1987). The results so far are not too promising. Primary two way MLR in randomly selected donor-acceptor pairs are highly variable. Reciprocal responses of donors in one- way MLR are usually unequal, suggesting immunogenetic differences (Caspi and Avtalion, 1984). We hoped that heterozygous gynogenetic offspring would show a segregation of alleles of a strong MLR

(MHC) locus as it did in allograft reactions (chapter 5). Unfortunately, the highly variable responses did not allow for a clear discrimination between responding and non-responding donor combinations (table 4).

Haemagglutination tests using specific allo-antisera are more promising for immunogenetic studies. Recently Kaastrup et al., (1989) succeeded in raising specific antisera against the allelic products of a putative MHC locus. The antisera were produced by immunising gynogenetic Dor-70 carp with peripheral blood leucocytes from gynogenetic siblings. The obtained antisera were operationally monospecific within the Dor-70 gynogenetic progeny. Flow-cytometer analysis of the cellular distribution of these histocompatibility antigens demonstrated their presence on erythrocytes, pronephros leucocytes and peripheral blood leucocytes (Kaastrup et al., 1989). In view of the previously noted fact that Dor-70 carp were homozygous for most biochemical markers, it is remarkable that this strain has retained its heterozygosity for a putative MHC locus.

Table 4 Stimulation indices (S.I.) of mixed leucocyte reactions between heterozygous gynogenetic siblings. (Komen, unpublished results).

		stimulator cells (fish nr)					
		1	2	3	4	5	6
responder	1	-	23.0	17.5	1.8	1.2	12.5
cells	2	4.1	-	26.6	3.2	0.2	6.1
(fish nr)	3	2.3	8.5	-	2.1	2.9	10.5
	4	0.9	3.2	3.4	-	1.1	1.0
	5	3.2	2.1	44.8	2.2	-	2.8
	6	2.7	25.8	92.5	3.3	2.0	-

Leucocytes from each fish were tested as responder or stimulator. Stimulator cells were irradiated with Co^{60} (30 Gy). Proliferation of responder cells was measured by ^{3}H-Thymidine incorporation. S.I. values were calculated as: $(1_{resp} + 2_{stim})/(1_{resp} + 1_{stim})$ (counts per minute).

During the course of this Ph-D study attempts were made to raise specific antisera within groups of gynogenetic offspring. Homozygous carp (EM group) were immunized with leucocytes from heterozygous gynogenetic siblings (2PB group). Three allo-antisera were obtained with haemagglutination titers between ^{2}log 4 and 6.

Table 5 Haemagglutination results of three different antisera on erythrocytes from half-sib (CO group), heterozygous gynogenetic (2PB group) and homozygous gynogenetic (EM group) siblings.

alloantisera	Co group		2PB group		EM group	
	+	-	+	-	+	-
EM_1 anti $2PB_1$	0	28	25	15	7	14
EM_2 anti $2PB_2$	19	9	39	1	16	5
EM_3 anti $2PB_3$	1	16	37	3	13	8

+ = agglutination; - = no agglutination. Antisera were obtained by repeated immunisations of homozygous gynogenetic fish (nrs 1, 2 and 3) with leucocytes from heterozygous gynogenetic siblings (1, 2 and 3; Komen, unpubl. results).

The haemagglutination test results are given in table 5. They are not readily interpreted in terms of a single locus with two allelic products segregating in homozygous combinations in the offspring. All three allo-antisera used were specific for the majority of fish from the 2PB group (the donor group), but less so for fish from the EM group (recipient group) or fish from a half sib control group. Absorptions with EM erythrocytes removed most reactivity. These results can be explained by assuming that the antigens within this strain are not polymorphic enough to be discerned by the carp antisera. However, the agglutination was not affected by treatment with ß mercapto-ethanol. This indicates that substances other than immunoglobulins might have been present in the produced antisera.

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Chapter 2

INTRODUCTION TO THE PAPERS

Summary

In this chapter a brief account of the meiosis and early development in common carp is presented, followed by a description of the genetic consequences of induced gynogenetic breeding. The chapter is concluded with an outline of the aims and structure of the present thesis. For the technical aspects of gynogenesis the reader is referred to reviews by Thorgaard (1983), Chourrout (1987) and Komen et al., (1990).

2.1 Meiosis and early development in common carp

Central to a discussion on the consequences and benefits of gynogenesis stands a thorough understanding of the meiotic processes and their timing in oocyte development and final maturation. The literature on this subject is often confusing.

In female common carp ovarian development starts with proliferation of germ cells or oogonia, 7-9 weeks after hatching (23 °C). These germ cells represent a stem cell population which gives rise to oogonia througout the life cycle of female carp (Parmentier and Timmermans, 1985; Wallace et al., 1987). Between 17 and 25 weeks after hatching the primordial gonad developes into an ovary. In this ovary many cysts are found containing either individual primary oogonia (16 μm), groups of secondary oogonia (i.e. after proliferation, 8-10 μm), or groups of early prophase oocytes (8μm). The oogonia contain a nucleus with a conspicious nucleolus and weak staining cytoplasma (haemaluin/eosin). The early prophase oocytes are characterised by a dense chromatine mass (Parmentier and Timmermans, 1985). Larger primary oocytes (pre-vitellogenic oocytes), surrounded by follicular cells, are observed in a later phase. In these oocytes numerous nucleoli are located along the nuclear periphery (peri-nucleolus stage). The cytoplasma stains distinctly basophilic. Meiosis is initiated during the transition from oogonium to primary oocyte. Each chromosome is duplicated (replicated) before meiosis. The chromosome and its copy remain together as *sister-chromatids* and behave as one functional unit during the following prophase I of meiosis. The first four stages of prophase I are:

(1) leptotene, (2) zygotene, (3) pachytene and (4) diplotene. The fifth stage of prophase, diakinesis, occurs later, after oocyte growth has been accomplished.

Leptotene: the chromosomes start to condense. The sister-chromatids appear as a single unit.

Zygotene: this stage is synonymous with the initiation of pairing or synapsis. The two homologous chromosomes align side by side. The resulting pair is called a "bivalent", but since each homologue consists of two sister-chromatids it is preferably called a *tetrad.*

Pachytene: as synapsis is completed, recombination nodules appear at regular intervals on the chromatids. At this stage *recombination* (crossing over) takes place (figure 1).

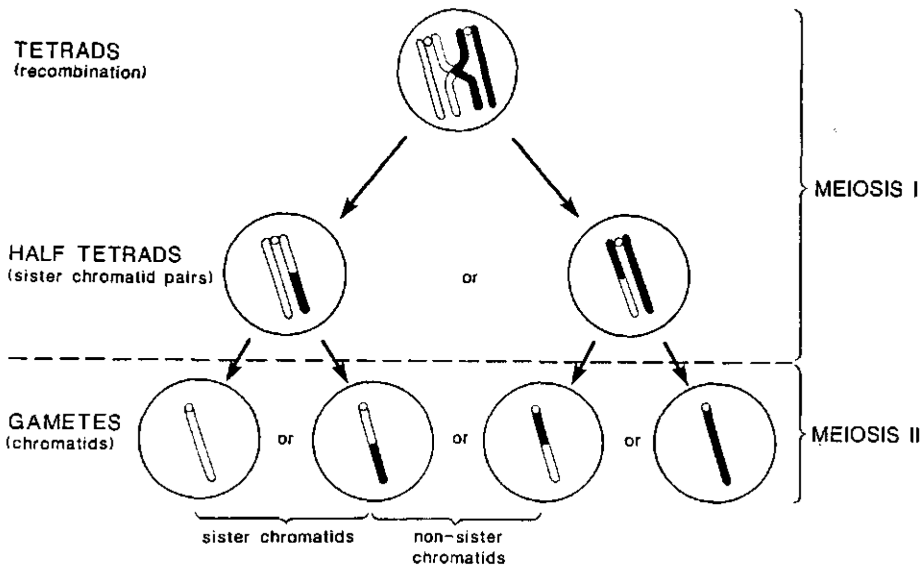
Diplotene: during desynapsis at the beginning of this stage, the homologues are pulled apart but remain attached at 1 or more recombination sites. These sites are termed *chiasmata* and are the morphological remnants of a cross-over event (Alberts et al., 1983). Meiosis is arrested at this stage and the oocytes start to grow. The tetrads decondense to form so called "lampbrush" chromosomes, and extensive RNA synthesis is commenced.

Leptotene, zygotene, and pachytene are collectively called the chromatin nucleolar stage in Teleosts (Tokarz, 1978; Wallace et al., 1987). Diplotene is often associated with the perinucleolus stage, but probably exists troughout the entire oocyte growth period: the characteristic lampbrush chromosomes disappear just before final oocyte maturation (Nagahama, 1983). The oocyte growth period is divided in two stages: cortical alveolar stage (yolk vesicle stage or endogenous vitellogenesis), and exogenous vitellogenesis (Wallace et al., 1987). In our carp stocks, these processes are initiated ± 6-8 months after hatching. Maturation and ovulation is first successful at an age of 15 months (25°C; Komen, unpublished results). Maturation is histologically visible as germinal vesicle migration (GVM) and germinal vesicle dissolution (GVD). During GVM the chromosomes condense and proceed from diplotene/diakinesis to metaphase I (Lessman and Kavumpurath, 1984). Meiosis I is completed during the following GVD with the extrusion of the first polar body. The tetrads are separated in two *half-tetrads* (sister-chromatid pairs) which are randomly divided between oocyte and polar body (Masui and Clarke, 1979; see also figure 1).

The oocyte immediately proceeds to metaphase II after which meiosis is arrested until ovulation and fertilization/activation. Egg activation is characterised by an initial increase in cytoplasmic free calcium, followed by a burst of cortical alveoli (Gilkey, 1981), extensive cytoplasma movement towards the animal pole, and resumption of meiosis. Fertilized but not activated eggs remain quiescent (Lessman and Huver, 1981), while activated but not

fertilized eggs do not develop beyond the initial meiotic division: the centrioles carried by the spermatozoid are required for mitotic spindle organisation (Alberts et al., 1983).
In properly fertilized and activated eggs meiosis is completed with the separation of the sister-chromatids during anaphase/telophase, and the expulsion of the second polar body. Again the sister-chromatids are randomly divided between the oocyte and the polar body (figure 1). The remaining chromatids, now called chromosomes, form a female pronucleus which fuses with the male pronucleus. The first mitotic division is initiated at 30 minutes after fertilization (25°C), and subsequent divisions follow with intervals of 20 minutes (Neudecker, 1976; see also chapter 4).

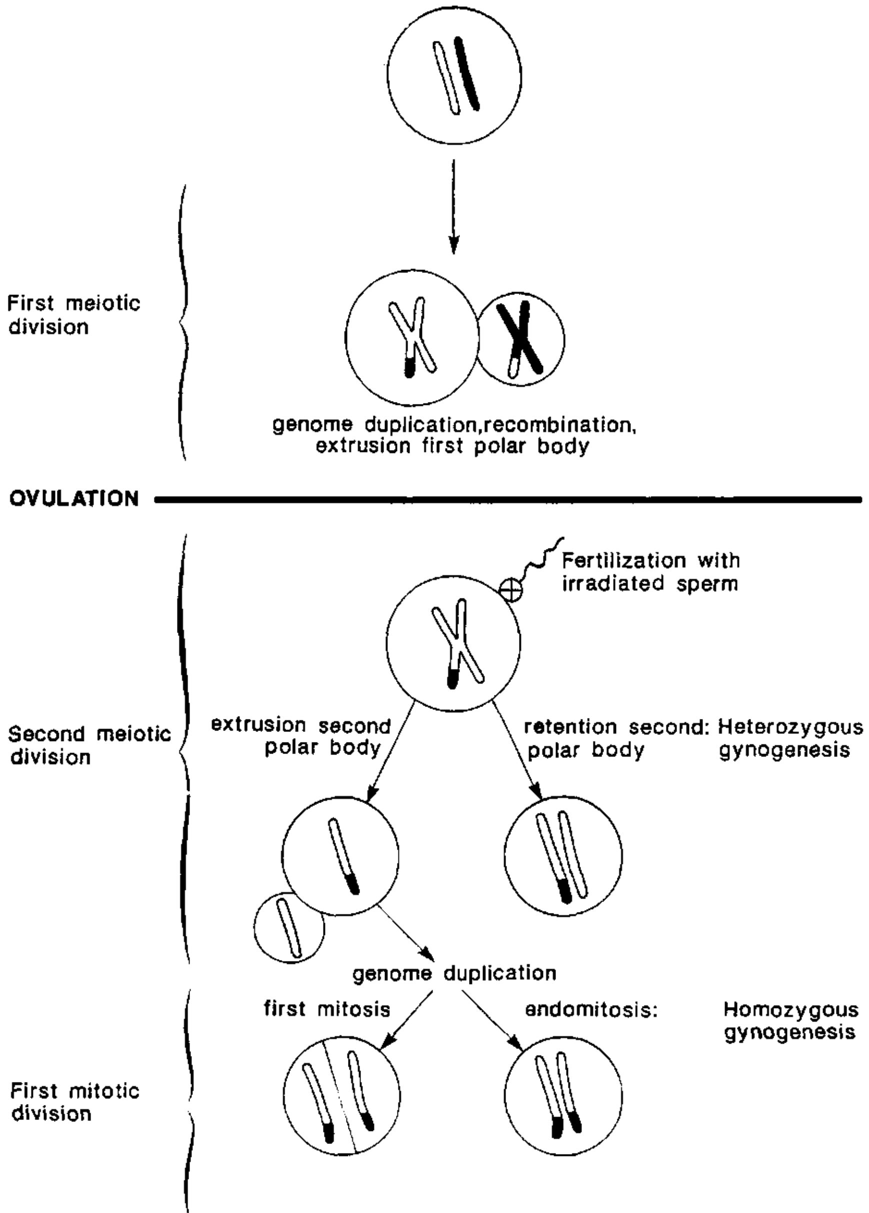
Figure 1 Chromosome configurations during meiosis.



2.2 Artificial gynogenesis

Gynogenesis requires the fertilization of a diploid egg with genetically inactivated sperm. This inactivation of sperm is usually achieved by gamma or U.V. irradiation (see Chourrout, 1987 and Komen et al., 1990, for details). Diploidy of the egg is achieved by inhibition of a meiotic or mitotic division.

Figure 2 The genetic consequences of fertilization with genetically inactivated sperm, and restoration of diploidy by inhibition of the second meiotic or first mitotic division (from Komen et al., 1990).



Following the scheme of events from the onset of meiosis to the first mitotic divion, three ways are open to intervention (figure 2):

- inhibition of meiosis I (endomeiosis)
- inhibition of meiosis II (retention second polar body)
- inhibition of mitotic division (endomitosis).Inhibition of meiosis I has not yet been induced artificially. It is however the most common mode in natural occurring gynogenetically reproducing triploid species. The best studied example is *Carassius auratus gibelio*, or crucian carp (In Japan a different subspecies is recognized: *C. auratus langsdorfii*). Crucian carp chromosomes replicate before meiosis, but synapsis and crossing-over does not take place. Apparently the entire meiosis I is omitted. The hexaploid oocytes go through one (the second) meiotic division after fertilization, in which the sister-chromatids of the three (!) homologous half-tetrads are separated and a triploid embryo is produced (Cherfas, 1966; Purdom, 1984). Since the sister chromatids are identical due to the absence of cross-overs, the resulting embryos are genetically identical (Nakanishi, 1987). The sperm, usually from a related species, i.e. common carp, activates the oocyte but does not decondense to form a pronucleus. Instead it is expelled from the oocyte (Yashimata et al., 1990).

Inhibition of meiosis II. This type of intervention is most commonly used to produce gynogenetic offspring since it is relatively easy to perform (see Nagy et al., 1978; Chourrout, 1987 and Komen et al., 1990). The principle is based on a disruption of the meiotic spindle by a physical shock administered to the egg. This shock can be a temperature shock (heat or cold) or a pressure shock. The microtubuli forming the meiotic spindle are destroyed in both cases. Applied just after fertilization and activation of the egg at metaphase II it results in an abortive meiotic division (retention of the second polar body). In carp, cold shocks can probably also cause a resorption of the polar body after extrusion (see chapter 3). The genetic consequences of this type of meiotic inhibition are profound. Since the sister chromatids are not divided, the egg is genetically identical to the group of half-tetrads left after meiosis I (figures 1 and 2). These half-tetrads will be homozygous (identical sister-chromatids) if crossing-over has not taken place: the resulting embryo is homozygous. However, if crossing-over does take place (and in most cases it does) then the sister-chromatids are not identical and the resulting half-tetrad will be heterozygous (figure 2). For a single locus with a normal gene A and a mutant gene a, the consequences are as follows (Nace

et al., 1970; Streisinger et al., 1986):

a) The resulting half-tetrads will become heterozygous if a single (or an uneven number of) cross-overs take place between the gene and its centromere. The genotype of the offspring is A/a.

b) the resulting half-tetrads will remain homozygous if no (or an even number of) cross-overs takes place between the gene and its centromere. The genotype of the offspring is A/A and a/a.

The frequency of heterozygous offspring A/a thus reflects the frequency of effective crossing-over between non-sister chromatids. This frequency is termed "Y". The frequencies of homozygous offspring are 0.5(1-Y) for A/A, and 0.5(1-Y) for a/a. The frequency of crossing over between gene and centromere diminishes when the gene is located closer to the centromere: "Y" is a measure for the gene-centromere distance (Nace et al., 1970). Table 1 lists some values of Y for different genes in common carp. The maximum value for Y under conditions of unlimited crossing-over between non-sister chromatids is 2/3 or 0.67 (Streisinger et al., 1986). The occurrence of genes with Y values > 0.67 indicate a high level of chiasma interference. Interference is considered a morphological constraint on the number of cross-overs and chiasmata that can occur on a chromosome. For common carp it means that a number of chromosomes have only one obligatory cross-over and one chiasma per meiotic event. The consequences of this phenomenon are extensively discussed in the following chapters.

Inhibition of the first mitotic division produces fully homozygous offspring. The genetic consequences of this type of gynogenesis are straightforward (figure 2). The haploid set of chromosomes in the ovulated and activated (fertilized) oocyte, are replicated (duplicated) prior to the first mitosis. Each chromosome then consists of two identical sister-chromatids which become separated during the following cell division. During endomitosis the sister chromatids are separated but cell division is skipped: the embryo becomes diploid.

The genotypes in the offspring are identical to the genotypes of the gametes (eggs). The variation in individual genotypes is considerable.

Let n be the haploid number of chromosomes in a species, then 2^n is the number of possible diploid genotypes which can be produced in the absence of crossing-over. This number is even larger with crossing-over.

Table 1 Frequencies of recombination for several genes in gynogenetic offspring of common carp.

Phenotype	Locus	Recombination Frequency (Y)	Ref.
Scalation	S/s	0.11 0.05	1,4
	N/n	0.97	1
Blond	B1/b1 ; B2/b2	0.12 ; 0.12	4
		0.42 ; 0.64	5
Transparent	+/tp	0.006	5
"pattern"	D/+	0.74	1
"pigment"	L/+	0.70	1
Esterase	S	0.09	2
	F	0.28	2
Transferrin	Tf	0.06, 0.05, 0.13	2,3
		0.06, 0.05, 0.14	5
Yellow eggs	+/ye	>0.90	5
"maleness"	+/mas-1	>0.90	5

(1- Cherfas, 1977; 2- Cherfas and Truveller, 1978; 3- Nagy et al., 1978; 4- Nagy et al., 1979; 5- Komen, unpubl. results).

The importance of this type of gynogenesis is illustrated by the second gynogenetic generation produced from such a homozygous gynogenetic female: this offspring is fully homozygous and identical, and can be considered as a clone. Such clones have only been produced in two aquarium fish species, *Brachydanio rerio* (Streisinger et al., 1981) and *Oryzias latipes* (Naruse et al., 1985).

2.3 Aim and structure of the present thesis

An important problem associated with scientific research on fish is the absence of well defined inbred strains. Inbred strains can be produced by conventional full-sib mating, but at least 10-15 generations are needed to produce homozygous inbred strains. Using females of common carp, which reach maturity at 1.5-2 years, this would take some 15-30 years. In practice experimental fishes are usually obtained from commercial fish farms, or bred in the laboratory using only few broodstock fish. In both cases the genetic background and the degree of inbreeding of the fishes is unknown. The experimental results from different laboratories are therefore sometimes difficult to compare. Another problem concerns the large variation in responses of individuals measured in endocrinological and immunological bioassay's. In consequence, large numbers of fish are usually needed to obtain statistically significant results.

In order to solve these problems, the aim of the present research was to develop homozygous inbred strains of common carp by gynogenetic breeding in only two generations. Such inbred strains would be of fundamental importance for ongoing basic and applied research on the immune response (Department of Experimental Animal morphology and Cell Biology) and sex differentiation and gonad development (Department of Fish Culture and Fisheries) of common carp.

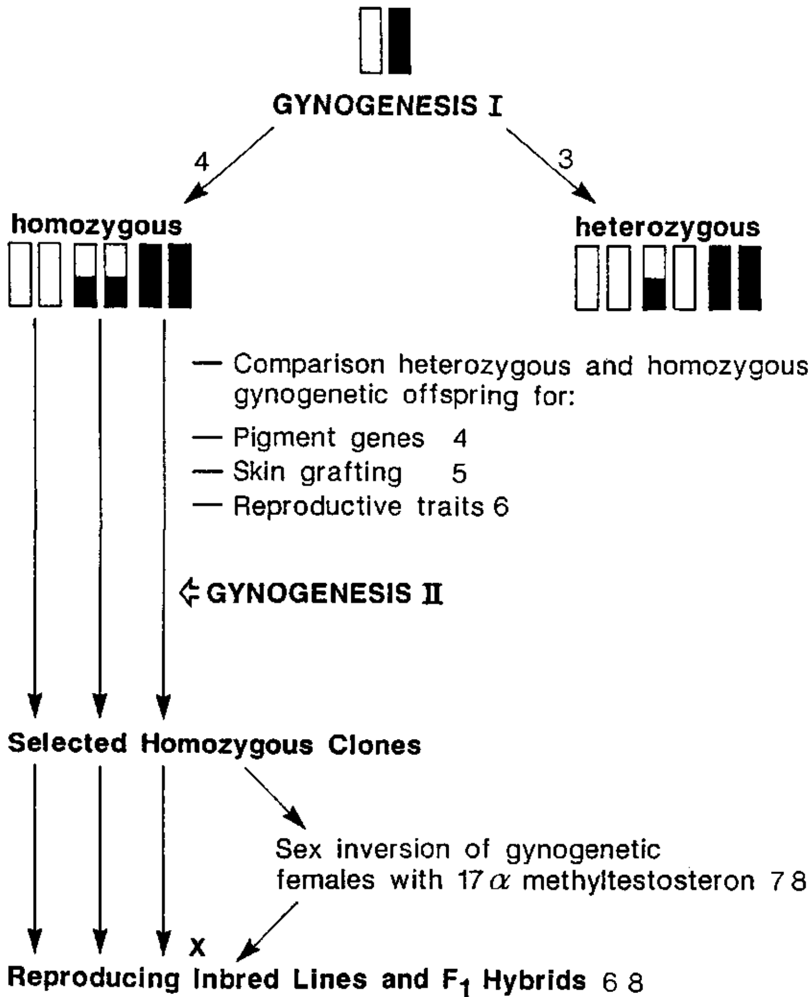
The structure of the present research is shown in figure 3.

In the first two chapters (3 and 4) the optimum conditions to produce gynogenetic fry by inhibition of the second meiotic or first mitotic division are investigated. Chapter 4 is concluded with the production of homozygous inbred strains by combination of both gynogenetic procedures.

Gynogenesis by inhibition of the first mitotis produces fully homozygous offspring. A first generation of homozygous offspring represents an entire library of all possible genotype combinations of the mother (if enough animals are kept) and each individual in such a library represents a potentially inbred strain. The selection of the homozygous individuals for subsequent gynogenetic reproduction should therefore be based on a thorough knowledge of the inheritance of various traits. Such knowledge includes the number of genes involved in the expression of a trait, their allelic interactions, the presence of deleterious recessives, the location of genes on chromosomes

and possible linkage associations with other genes. This information can be obtained by comparing heterozygous and homozygous gynogenetic sibling offsprings. A comparison of skin graft reactions in full-sib, heterozygous and homozygous gynogenetic siblings is described in chapter 5. The gonad development and fertility of such groups was investigated in chapter 6.

Figure 3 Thesis structure (numbers refer to thesis chapters)



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Since gynogenetic inbred strains are expected to be all female, a method for effective sex inversion had to be developed to produce inbred strains with functional males. The effects of oral administration of testosterone on gonad development of non-inbred carp are described in chapter 7. The effects of testosterone administration on gonad development of fish from two inbred strains and 4 F1 hybrids are described in chapter 8. The results are presented in the context of various observations concerning atypical sex ratio's in non-inbred and gynogenetic common carp. Experiments, designed to elucidate the genetic basis of atypical sex ratio's, are also described in this chapter.

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Chapter 3

GYNOGENESIS IN COMMON CARP (CYPRINUS CARPIO L.) I: EFFECTS OF GENETIC MANIPULATION OF SEXUAL PRODUCTS AND INCUBATION CONDITIONS OF EGGS

J. Komen, J. Duynhouwer, C.J.J. Richter and E.A. Huisman

(Aquaculture 69: 227-239)

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Gynogenesis in Common Carp (*Cyprinus carpio* L.) I. Effects of Genetic Manipulation of Sexual Products and Incubation Conditions of Eggs

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(Accepted 4 August 1987)

ABSTRACT

Komen, J., Duynhouwer, J., Richter, C.J.J. and Huisman, E.A., 1988. Gynogenesis in common carp (*Cyprinus carpio* L.). I. Effects of genetic manipulation of sexual products and incubation conditions of eggs. *Aquaculture*, 69: 227-239.

The effects of genetic manipulations of eggs and sperm and incubation conditions of eggs on the yields of gynogenetic fry were investigated. Ten ml of sperm (1:3 diluted) was inactivated using UV-irradiation at a dose of 2200 J m^{-2} min^{-1} for 1 h. Gynogenesis was achieved by cold-shocking eggs, fertilized with irradiated sperm, at different times after fertilization. Consistent yields of 25-50% viable, gynogenetic fry were obtained when eggs were incubated at 24°C and cold-shocked (0°C, 45 min) 1-2 or 7-9 minutes after fertilization. This bimodal response of eggs to cold shocks was essentially different from the responses found by other authors who researched gynogenesis in carp using NaCl/urea solutions and temperatures below 24°C for incubation. Although the latter conditions proved to delay the first sensitive period, this could not fully account for the observed differences.

INTRODUCTION

In artificial gynogenesis, eggs are fertilized with irradiated sperm, which is genetically inactive, and kept diploid by suppression of the second meiotic division (retention of the second polar body) or of the first mitotic division. In the first case the degree of homozygosity depends on the rate of crossing-over between non-sister chromatids during the first meiotic division (Nagy et al., 1979; Thompson, 1983; Chourrout, 1984, 1986). In the second case completely homozygous diploid offspring are produced (Streisinger et al., 1981; Nagy, 1986).

Genetic inactivation of the sperm has been achieved by irradiation with ^{60}Co gamma or UV rays (Table 1). Thermal or pressure shocks are used for the

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TABLE 1

Methods of irradiation of sperm samples of common carp, *Cyprinus carpio* L.

Dilution	Irradiation	Conditions	Viability control	Reference
-	^{60}Co; 100 krad; 5 krad/min	on ice	-	Cherfas, 1975
-	^{60}C; 100 krad; 10.9 krad/min	on ice	-	Nagy et al., 1978
-	^{60}C; 100 krad/-	on ice	-	Gervai et al., 1980b
-	^{60}C; 1000 Gy	on ice	-	Linhart et al., 1986
-	UV; 1 mW/cm^2; duration 15 min	stirred/on ice	motility check	Stanley, 1976
1:40/10 ml	UV; 300 erg/mm^2	-	-	Tsoy, 1981
1:9/2.5 ml	UV; sperm-lamp distance 4 cm, duration 3-4 min	stirred	motility check	Hollebecq et al., 1986
1:9/10 ml	UV; 200 J m^{-2} min^{-1}; 5-60 min, sperm-lamp distance 2.5 cm	stirred/on ice	fertilization[a]/motility check	Present paper

[a]Fertilization rate was estimated from the survival of eggs after 24 h, fertilized with irradiated sperm, compared to the survival of eggs after 24 h, fertilized with untreated sperm.

TABLE 2

Methods of genetic manipulation of eggs of common carp, *Cyprinus carpio*. Retention of the second polar body (2pb) in combination with fertilization with irradiated and untreated sperm can result in gynogenesis and triploidy, respectively. Gynogenesis can also be achieved by fertilization with irradiated sperm and suppression of the first mitotic division (1md)

Incubation temperature (°C)	Degumming of eggs	Shock timing (min) after fertilization			Shock temp./duration (°C/min)	Yield of viable fry (% of incubated eggs)	Reference
		Gynogenesis		Triploidy			
		2pb	1md	2pb			
20	+	5/15	-	-	4/60	1.1-31/0.9-56%	Nagy et al., 1978
20	+	-	-	1-9	0/45	max. 50%[a]	Gervai et al., 1980a
23	−	-	-	5	0/30	5-10%[a]	Ueno, 1984
?	−	3-5	-	3-5	0-4/30	36%/?	Wu et al., 1986
23	+	15	-	-	0/60	0.5%	Van Muiswinkel et al., 1986
?	+	5/15	-	-	0-4/60	0.02-1.04/0.13-2.86%	Linhart et al., 1986
20	−	3-5	-	-	39/2	max. 50%	Hollebecq et al., 1986
24	−	1-2/7-9	-	-	0/45	25-53/23-54%	Present paper
22	+	-	40	-	40/2	max. 10.5%	Nagy, 1986
24	−	-	30	-	40/2	5-18%	Komen et al., in prep.

[a]Percentage of triploidy unknown.

suppression of the second meiotic or first mitotic division of the oocytes. In common carp, *Cyprinus carpio*, gynogenesis has been induced using these methods but the yields of viable gynogenetic fry were highly variable, ranging from 1 to 50% (Table 2). Differences in genetic manipulation techniques and incubation conditions of eggs may account for these variable results.

The establishment of gynogenetic carp inbred broodstock lines in our laboratory aims at standardization of endocrinological (Richter et al., 1987) and immunological (Van Muiswinkel et al., 1986) bio-assays. In the present paper the effects of genetic manipulations and incubation conditions of eggs are investigated to maximize yields of gynogenetic fry under standardized conditions.

MATERIALS AND METHODS

Husbandry of broodstock and fry

Broodstock of common carp was raised from eggs to maturity in the hatchery of the Department of Fish Culture and Fisheries at Wageningen Agricultural University. The fish were kept in rectangular tanks, containing 800 l of water at 23°C. The flow rate was 20 l/min, maintaining the O_2 content above 7 ppm.

The fish had reached an age of about 1.5 years (mean weight 2 kg) at the time that they were used for artificially induced breeding. They were fed trout pellets (Trouvit, The Netherlands) at a daily ration of 1% of body weight using Scharfflinger conveyerbelt feeders.

Fry were fed *Artemia salina* nauplii during the first 2 weeks after hatching, followed by trout pellets (Trouvit-00) according to the recommendations of Huisman (1976).

Irradiation of milt, cold shocking of eggs and determination of incidence of gynogenetic fry

Eggs and milt were obtained by artificial reproduction as described by Woynarovich (1962). Females, homozygous for a gene determining scalation (mirror, ss), received two injections of carp pituitary suspension (cPS) at 0.3 and 3 mg acetone-dried carp pituitary (Hydroquest International, Rosemont, NY) per kg body weight, respectively.

The time interval between the two injections was 30 h and stripping of eggs was carried out 10–11 h after the second injection. Males, heterozygous for scalation (scaled, Ss), received one injection of 1 mg cPS per kg of body weight and stripping of milt was carried out 16 h afterwards. The motility of the sperm was checked under a microscope (Zeiss, 100×) by adding some water to a few droplets of milt (control of sperm quality). The milt stock was diluted 1:3 with 0.85% NaCl solution to prevent sperm motility. Samples of 10 ml were spread on a large watch-glass (in order to obtain a thin layer of spermatozoa) and

placed in a petri-dish filled with ice. The milt was magnetically stirred during irradiation. The lamp (Philips 15 watt germicidal UV tube, 253.7 nm) was warmed up 30 min before the onset of irradiation. The distance between the lamp and the sperm sample was 2.5 cm and the intensity of irradiation, measured at the sperm surface, 2200 J m^{-2} min^{-1} (Photodyne optical power energy meter, model 66XLA with cut-off filter WG 305 and fused-silica neutral density filter).

Samples of 200–400 eggs were mixed with 100 μl of irradiated sperm (control of sperm irradiation) or with untreated sperm (control of egg quality). The moment of water addition (at 24°C) to the mixture of eggs and milt was taken as the fertilization time ($t=0$). The eggs were spread on a screen bottom (mesh size 0.5 mm) of a basket (PVC, diameter 10 cm), which was placed in a thermoregulated (24°C) water recirculation system.

Cold shocking was done at different times after fertilization and for various lengths of time by transferring the basket to a tank with pre-cooled water at the desired temperature.

The gynogenetic effects of the manipulations were checked by fertilizing untreated and cold-shocked eggs with irradiated sperm. When there were nonviable fry in the first and normal viable fry in the second treatment, the manipulations were considered to have been effective. Four weeks after hatching, scalation of fry originating from cold-shocked eggs fertilized with irradiated sperm was also assessed. A complete absence of scalation was considered to be a reliable second check that no paternal transmission of genes had occurred.

Experimental design

All experiments except the last were carried out in duplicate. One male and one female broodfish were used per experiment.

In the first experiment the effects of dilution (1:3 and 1:9 with physiological saline solution) and duration of irradiation (0, 5, 10, 20, 30, 40, 50 and 60 min) on genetic inactivation and mortality of sperm were examined.

The second experiment concerned the effects of cold shocking eggs at various times after fertilization. Eggs were incubated at 24°C and cold shocked (0°C, 45 min) 0, 0.5, 1, 2, 3, 5, 7, 9, 12 and 15 min after fertilization. Time and duration of the cold shock were based on the results of Gervai et al. (1980a) and Nagy et al. (1978). Sperm (dilution 1:3) was irradiated for 60 min (see first experiment).

In the third experiment the effects of different cold shock temperatures (0, 4 and 8°C) and durations (15, 45 and 90 min) were examined. Eggs were incubated at 24°C and exposed to a cold shock given 1 min after fertilization. One minute was chosen because of the high yields obtained in the second experiment. All nine combinations were tested at the same time. For inactivation

of sperm (see second experiment) a dilution of 1:3 and an irradiation duration of 60 min was applied.

In the fourth experiment the influence of incubation temperature prior to the onset of the cold shock was investigated. Eggs were incubated at three different temperatures (24, 22 and 20°C) and cold shocked (0°C, 45 min) at various times (1, 2, 3, 4, and 5 min) after fertilization. After cold shocking they were incubated at 24°C. Sperm was diluted 1:3 and irradiated for 60 min.

In the fifth experiment the effects of an NaCl/urea-solution (commonly used to remove the sticky layer of carp eggs) on the yields of viable gynogenetic fry were investigated. According to Woynarovich (1962), this solution increases the fertilizing capacity of sperm and extends the period during which the eggs can be fertilized. Eggs were fertilized and incubated using either a 0.4% urea/0.3% NaCl solution or normal water, both at 24°C. The eggs were then cold shocked (0°C, 45 min) at various times (1, 2, 3, 5, 7, and 9 min) after fertilization. Sperm was diluted 1:3 and irradiated for 60 min.

Parameters and statistical analysis

The survival of developing eggs (%) at $t=24$ h and $t=48$ h (only in the first experiment) and of viable fry at $t=96$ h were used as parameters to study the effects of genetic manipulation and incubation conditions. All data, except those from the fifth experiment, including the controls of sperm irradiation and egg quality, were transformed using an arcsin transformation (Sokal and Rohlf, 1969) and analysed with Duncan's multiple range test ($P=0.05$) using an SPSS computer program (Nie et al., 1975).

RESULTS

The controls for egg quality in all experiments had high rates of normal viable fry (85–95%), indicating a good quality of eggs and sperm. No scaled individuals were found amongst the presumed gynogenetic fry in any of the experimental groups 4 weeks after hatching, indicating the absence of transmission of paternal genes in the fry.

First experiment (Fig. 1)

At $t=24$ h there was no statistically significant effect of irradiation duration of sperm diluted 1:3 (compare $t=0$ and 60 min) on the survival rate of embryos ($P=0.05$). At $t=48$ h, however, the survival rate decreased significantly during the first 20 min of irradiation and remained fairly constant in the following 30 to 60 min. The yield of normal fry at $t=96$ h decreased significantly with increasing duration of irradiation. The lowest values were found at durations of 50 and 60 min.

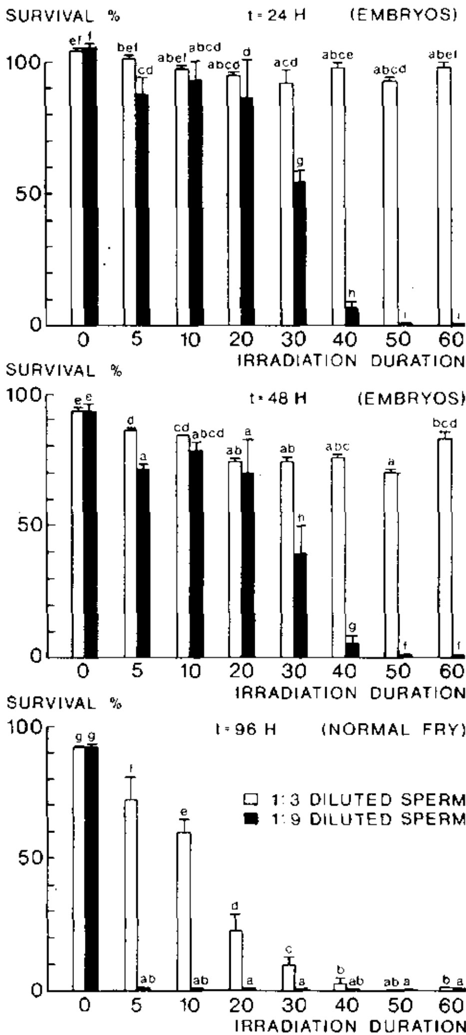

Fig. 1. Effects of dilution (1:3, and 1:9) and duration of irradiation (min) on genetic inactivation of carp sperm, expressed by survival rate of developing eggs and yield of normal fry. Irradiation dose 2200 J m^{-2} min^{-1}. Incubation temperature 24°C. Means with a common superscript are not significantly different by Duncan's multiple range test ($P=0.05$).

At a sperm dilution of 1:9 and at $t=24$ and 48 h, a significant decrease of survival of embryos was found between 20 and 40 min of irradiation. Complete mortality was observed at durations of 50 and 60 min. The additional control for sperm quality at $t=0$ revealed that at these irradiation durations the sperm became immotile. At $t=96$ h a very high mortality occurred irrespective of the duration of irradiation.

In the following experiments a dilution of 1:3 and an irradiation duration of 60 min was applied.

Second experiment (Fig. 2)

At $t=24$ h the survival of embryos from eggs cold shocked at $t=1$, 7 and 9 min was significantly higher than that from eggs cold shocked at $t=0.5$, 2, 5, 12 and 15 min. ($P=0.05$). Shocks administered at $t=0$ and 3 min resulted in 100% mortality.

At $t=96$ h the yield of normal fry from eggs cold shocked at $t=1$, 2, 7 and 9 min was significantly higher than that from eggs cold shocked at $t=0.5$ and 5 min. Shocks administered at $t=0$, 3, 12 and 15 min resulted in 100% mortality. There was a low survival in the sperm irradiation control (2%) indicating that almost all fry can be considered to be of gynogenetic origin.

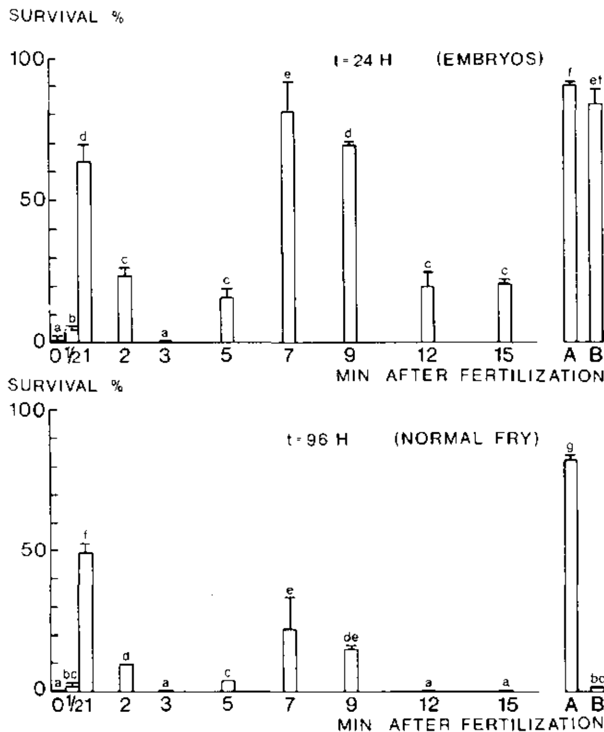

Fig. 2. The effects of cold-shocking eggs at various times after fertilization on the survival of embryos after 24 h and the yield of normal fry after 96 h. Cold shock 0°C, 45 min. Incubation temperature 24°C. A = control of egg quality. B = control of sperm irradiation. Means with a common superscript are not significantly different by Duncan's multiple range test ($P=0.05$).

Third experiment (Fig. 3)

At $t=24$ h the survival of embryos from eggs which were cold shocked for 15 min was high (82% at 8°C). Long shock durations (45 and 90 min) in combination with high temperatures (4°C and 8°C) caused high mortalities. The survival rates were significantly different from those of the other cold-shocked egg samples and controls ($P=0.05$).

At $t=96$ h the survival of normal fry from eggs cold shocked at 4° and 8°C was significantly lower than that from eggs treated at 0°C. A high survival, irrespective of shock duration, occurred at 0°C. There was a low survival in the control for sperm irradiation (0.3%).

Fourth experiment (Fig. 4)

At $t=24$ h there was no incubation temperature effect on egg samples cold shocked at $t=1$ and 2 min. A significant influence of incubation temperature

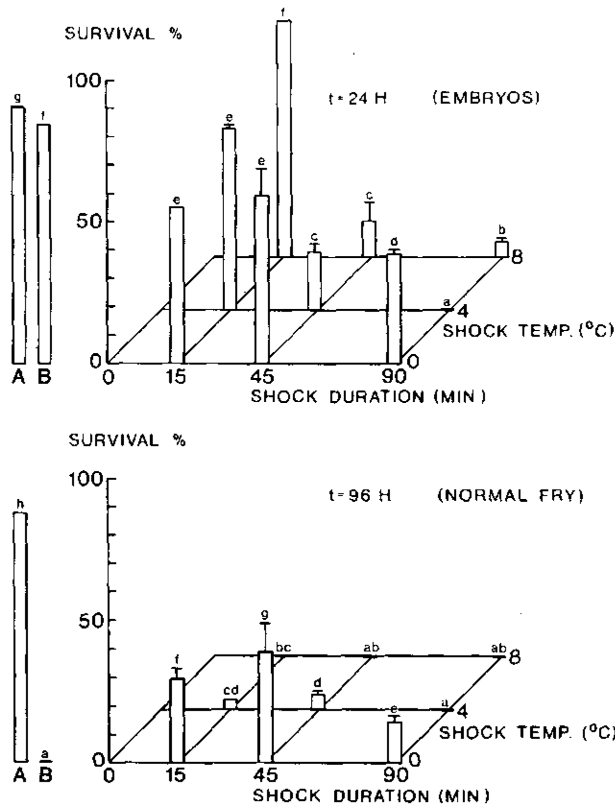

Fig. 3. The effects of different combinations of cold shock temperature and duration on the survival rate of embryos and the yield of normal fry. The eggs were shocked 1 min after fertilization. Incubation temperature 24°C. For further explanation see Fig. 2.

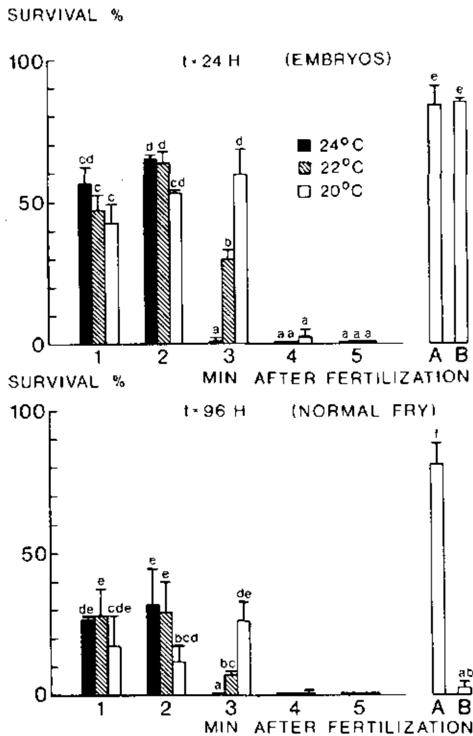

Fig. 4. The effects of different incubation temperatures prior to cold shocking on the survival of embryos (after 24 h) and normal fry (after 96 h). The cold shock was administered at various times after fertilization. Cold shock 0°C, 45 min. Incubation temperature 24°C. For further explanation see Fig. 2.

on survival became apparent in egg batches cold shocked at $t=3$ min, survival being lowest at 24°C and highest at 20°C ($P=0.05$). Irrespective of incubation temperature, cold shocks administered at $t=4$ and $t=5$ min caused high mortalities.

At $t=96$ h a similar pattern of effects of incubation temperature on the survival of fry was found. The only exception was a significant difference between egg samples incubated at 20 and 22°C and cold shocked at $t=2$ min. The yield of normal viable fry in the sperm irradiation control was 3.2%.

Fifth experiment (Fig. 5)

At $t=24$ and 96 h the survival of embryos and fry from eggs cold shocked at $t=1$, 2, and 7 min, using water as a fertilization medium, was relatively high. High mortalities of fry occurred with cold shocks applied at $t=3$, 5 and 9 min.

The egg samples which were fertilized in NaCl/urea, and cold shocked between 3 and 9 min, showed high survival rates after 24 h. Survival of fry oc-

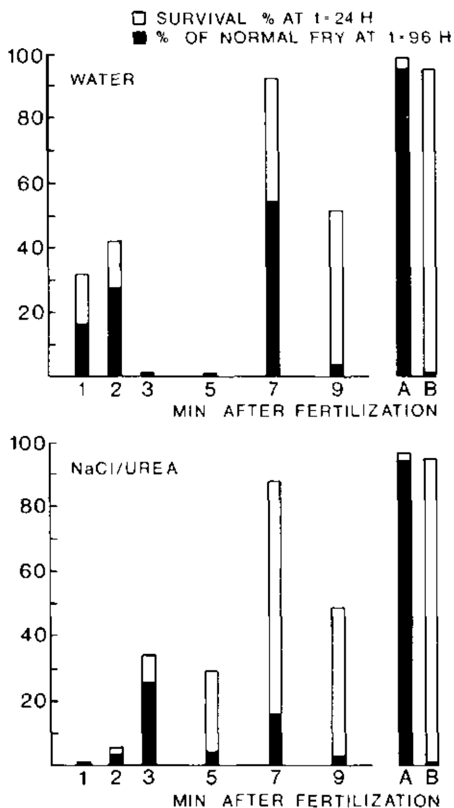

Fig. 5. The effects of NaCl/urea solution, used for fertilization and incubation prior to cold shocking, on the survival of embryos and yields of normal fry from eggs cold shocked at various times after fertilization. Cold shock 0°C, 45 min. Incubation temperature 24°C. For further explanation see Fig. 2.

curred at the shock interval of 2–9 minutes, with relatively small peaks at $t=3$ and 7 min.

The sperm irradiation controls showed very low values and in this respect there was no difference between the two fertilization media.

DISCUSSION

It has been stated that UV irradiation permits only small samples (2.5 ml or less) of sperm to be treated at the same time (Thorgaard, 1983; Chourrout, 1986).

In the first experiment complete genetic inactivation was achieved using a relatively high density of sperm (dilution 1:3) in combination with a long duration of irradiation (60 min). Genetic inactivation was also achieved using a

low density of sperm (1:9 dilution) and short durations of irradiation (5–40 min). These conditions correspond with those used by Stanley (1976) and Hollebecq et al. (1986) (Table 1).

Irradiation of sperm diluted 1:9 for 50 and 60 min in the present research resulted in immotile sperm with corresponding 100% mortality (within 24 h) in the egg samples fertilized with this sperm. In contrast, irradiation of sperm diluted 1:3 for 50–60 min resulted in a high survival of embryos up to hatching. Many embryos and hatched fry displayed the typical haploid syndrome, i.e. microcephalia, short and curved tail and distorded and elongated yolk sac (Gervai et al., 1980b). The difference in survival between sperm diluted 1:9 and 1:3 might be caused by the greater penetration of UV in more dilute sperm samples.

Our results do not show the classic Hertwig effect, i.e. low survival of embryos at low doses of irradiation and high survival at higher doses. According to Ijiri and Egami (1980), this effect is only seen at very low doses of irradiation, 50 J/m^2 or less.

Cold shocks (at 4°C, for 60 min) administered at 5 and 15 min after fertilization to eggs incubated in an NaCl/urea solution (at 20°C) resulted in yields of gynogenetic fry of 1.1–31 and 0.9–56% respectively (Nagy et al., 1978; Table 2). First and second sensitive periods at 1–3 and 7–9 min after fertilization were also found in our research (second experiment).

An incubation temperature below 24°C (fourth experiment) and the use of NaCl/urea solution (fifth experiment) both delay the first sensitive period a little; but this fails to give a full explanation for these differences. The differences in time sequence of the two sensitive periods between the two research works may reflect differences in husbandry conditions or differences in strains. The broodfish used by Nagy et al. (1978) were kept in ponds and were of local Hungarian and Yugoslavian strains. Those used in our experiments were raised from eggs to maturity under standardized hatchery conditions and were of a Dutch strain.

Applying cold shocks at a fixed time after fertilization without determining the time sequence of the two sensitive periods under the conditions used (incubation temperature, fertilization media) may account for the low yields of gynogenetic fry mentioned in other publications (Ueno, 1984; Linhart et al., 1986; Van Muiswinkel et al., 1986). The effects of different cold shock temperatures and durations, applied during the first sensitive period, revealed that a relatively high survival, irrespective of durations (15–90 min), occurred at 0°C (third experiment). In contrast to Nagy et al. (1978), we found that a shock temperature of 4°C in combination with a long shock duration resulted in a low yield of gynogenetic fry.

The bimodal response to cold shocks seems to be typical of carp eggs. In many other species, e.g. *Tilapia aurea* (Valenti, 1975), *Siluris glanis* (Krasnai and Marian, 1986) and *Clarias gariepinus* (Richter et al., 1986) only one sen-

sitive period was found. The bimodal response in carp can probably be related to two consecutive phases occurring during meiosis. Temperature and pressure shocks dissociate the microtubules that make up the spindle during meiosis II (Dustin, 1984). Shocks applied at a later stage of second polar body formation caused an absorption of the second polar body by the ovoplasma (Makino and Ozima, 1943; Romashov and Belyaeva, 1965; Rott, 1965).

The results of Hollebecq et al. (1986) using heat shocks agree with our results in those few cases where two sensitive periods were found. The majority of their results, however, revealed only one sensitive period, variable in its location (Table 2). Heat shocks therefore may act in a different way on the eggs than cold shocks.

Gynogenesis by inhibiting the first mitotic division (Table 2) was recently achieved in carp by heat shocking (40°C) eggs at 30 (Komen et al., in preparation) or 40 (Nagy, 1986) min after fertilization. The homozygous fish obtained by this method will be used to develop a gynogenetic inbred broodstock line of common carp.

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Chapter 4

GYNOGENESIS IN COMMON CARP (*Cyprinus carpio L.*) II: THE PRODUCTION OF HOMOZYGOUS GYNOGENETIC CLONES AND F_1 HYBRIDS

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(Aquaculture, in press)

ABSTRACT

Homozygous gynogenetic fry of common carp (*Cyprinus carpio L.*) were produced by heat shocking eggs, activated with U.V.-irradiated sperm (1:3 diluted, 10 ml, 2200 J/m2, min) during metaphase of the first mitosis. Consistent yields of 5 - 15 % viable, gynogenetic fry were obtained when eggs were shocked at 40 °C for 2 minutes, 28-30 minutes after fertilization. The homozygous nature of the gynogenetic fry was demonstrated by the mendelian segregation patterns of three recessive mutant pigment genes. Homozygous inbred strains were produced by heterozygous gynogenetic reproduction (2nd polar body retention) of homozygous gynogenetic females, while F_1 hybrids were produced by crossing these females with homozygous gynogenetic male siblings. The clonal nature of these strains was unequivocally demonstrated by the acceptance of reciprocally exchanged skin allografts.

INTRODUCTION

Inbred strains are important instruments in immunological, endocrinological and genetic studies. In fish, conventional inbred strains have only been developed in two species: Xiphophorus maculatus (Kallman, 1970) and *Oryzias latipes* (Hyodo-Taguchi, 1980), while inbred strains of commercially important fish species are still lacking. Most Salmonidae and Cyprinidae have long generation intervals and full-sib mating is therefore not a method of choice (Falconer, 1981). For these species gynogenesis has been adopted as a rapid method for the production of inbred strains (Thorgaard, 1983; Nagy and Csanyi, 1984). In gynogenesis, eggs are fertilized with irradiated and therefore genetically inactive sperm, and made diploid by either retention of the second polar body (heterozygous gynogenesis) or by inhibition of the first mitosis (endomitosis or homozygous gynogenesis).

Studies on the degree of inbreeding in heterozygous gynogenetic offspring revealed a considerable degree of heterozygosity for several loci due to a high rate of recombination between these loci and the centromere during meiosis (Thorgaard et al.,1983; Thompson and Scott, 1984). Repeated heterozygous gynogenetic reproduction

will therefore lead to genetically identical but only partly homozygous strains (Nagy and Csanyi, 1984). In contrast homozygous gynogenesis should produce fully homozygous offspring, and gynogenetic reproduction of such homozygous animals would thus produce homozygous inbred strains in only two generations, as has been demonstrated in *Brachydanio rerio* (Streisinger et al., 1981) and *Oryzias latipes* (Naruse et al., 1985). Such inbred strains are often termed clones.

In our laboratory, we are interested in the development of homozygous inbred strains of common carp (*Cyprinus carpio*) to use in studies on the genetics of the immune response (Kaastrup et al., 1989; Komen et al, 1990) and sex differentiation (Komen and Richter, 1990),

In a previous paper we investigated the genetic inactivation of carp sperm by U.V.-irradiation and the production of heterozygous gynogenetic common carp under standardized conditions (Komen et al., 1988). In this paper we describe the optimum conditions to produce homozygous gynogenetic fry by inhibition of the first mitosis through heat shocks. We also report on the production of homozygous inbred strains by subsequent gynogenetic reproduction through retention of the second polar body. The clonal nature of these strains was confirmed by skin transplantations.

MATERIALS AND METHODS

Husbandry of broodstock and fry.

The broodstock of common carp used for gynogenetic experiments was a F1 generation from a cross D1(♂) x W15(♀).

D and W stands for random bred German (D) and Dutch (W) carp strains. The broodstock was raised from egg to maturity in the hatchery of the central fish culture facilities at the Wageningen Agricultural University. Fry were raised at 25°C and fed freshly hatched Artemia salina nauplii during the first three weeks after hatching, followed by vitamin-C enriched trout pellets at a daily ration of 30 gr/$Kg^{0.8}$ body weight. Mature fish were kept in 800 l rectangular tanks with recirculating water (23 °C), and were fed trout pellets (Trouvit, Trouw, The Netherlands) at a daily ration of 1 % of body weight, using conveyer-belt feeders. The flow rate was 20 l/min, maintaining the

O_2 content above 5 ppm. The broodstocks had reached an age of 1.5 years (mean weight 2 kg) when they were used for artificially induced breeding.

Irradiation of milt and temperature shocking of eggs.

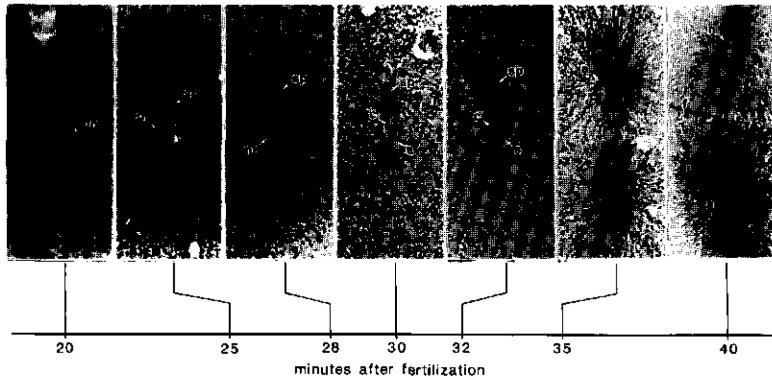
Eggs and milt were obtained and treated as described previously (Komen et al., 1988). Females received two consecutive injections of carp pituitary suspension (cPS: Hydroquest International, Rosemont, N.Y) of 0.3 and 3 mg per kg body weight respectively, and were stripped 10 - 11 hrs after the last injection (at 23 °C). Males received one injection of 1 mg cPS per kg body weight and were stripped 16 hrs later.
The milt stock was diluted 1:3 with ice-cold 0.85 % NaCl solution and irradiated with U.V. (Philips 15 W germicidal tube, 253.7 nm) for 60-65 min. The intensity of irradiation, at a distance of 2.5 cm between lamp and sperm surface, was 2200 $J/_{m2,min}$.
Samples of 400 - 800 eggs were mixed with 200 μl of irradiated milt suspension and activated by adding water. The moment of water addition was taken as the fertilization time t_0. The eggs were spread on the screen bottom (mesh size 0.5 mm) of a round basket ((/o 10 cm, transparent P.V.C), which was placed in a thermo-regulated water recirculation system (24.0 °C). The system and baskets had been pretreated with 5 ppm malachite-green to prevent fungus infection.
Heat shocking was done by transferring the baskets to a tank with pre-heated water at the desired temperature. The controls for sperm irradiation and egg quality were not heat shocked.
The quality of the gametes was checked by the survival of embryo's and yields of normal fry from eggs fertilized with non-irradiated milt and incubated without a temperature shock (control for egg quality). The effectiveness of sperm irradiation was asessed by the absence of normal, viable fry in groups of eggs fertilized with irradiated milt but incubated without a temperature shock (control for sperm irradiation).

Determination of the optimum heat shock treatment to produce homozygous gynogenetic fry.

The first mitotic division in carp embryos was located by histological examination of embryos, fixed at regular intervals after fertilization. Preliminary heat shock experiments

had indicated this mitotic division to occur between 20 and 40 minutes after fertilization. Samples of 40-80 eggs were mixed with undiluted non-irradiated milt in petri dishes (o/5 cm) and incubated in a thermo-regulated (24°C) water bath.

Figure 1 First mitotic division and initiation of cleavage in embryo's of common carp (*Cyprinus carpio L.*)



t 20 fusion of male and female pronuclei

t 25 prophase: pronuclei with spindle pole formation and condensation of chromosomes

t 28 prometaphase: breakdown of nuclear envelope and arrangement of chromosomes in spindle

t 30/t 32 metaphase: clear bipolar spindle with chromosomes aligned across the centre

t 35 late anaphase: ordered segregation of chromosomes and increasing aster size

t 40 telophase: formation of daughter nuclei and progression of cleavage furrow

pn = pronuclei; sp = spindle pole; ne = nuclear envelope; s = spindle; c = chromosomes; a = aster; f = cleavage furrow; n = daughter nuclei. (x 740)

The developing embryos were fixed at t_{20}, t_{25}, t_{28}, t_{30}, t_{32}, t_{35} and t_{40} min after fertilization in Bouin's fluid. Groups of 10 -14 eggs from each sample were embedded in paraffin and serially sectioned at 14 μm. The sections were stained according to Lessman and Huver (1981) in Harris-hematoxilin / congo-red.

In a consecutive experiment, the effects of heat shocking eggs at different times after fertilization around the time of first mitosis were examined. The eggs were incubated at 24.0 °C and heat shocked (40.0 °C, 2 min) at 2 min intervals between 24 and 40 minutes after fertilization. Temperature and duration of the heat shock were based on the results of Streisinger et al. (1981) and Nagy (1987). In total 6 trials were performed, each with a different male and female. Another experiment concerned the effects of different combinations of heat shock temperature (39, 40 and 41 °C) and duration (1, 2, and 3 min). Eggs were incubated at 24.0 °C and heat shocked at 30 min after fertilization. For this experiment 1 male and 1 female were used.

All treatments in both experiments were carried out in duplicate. The treatments were considered to have been effective if the heat shocked groups produced significantly more normal fry than the control groups for sperm irradiation.

Determination of incidence of homozygous gynogenetic fry.

The homozygous nature of the gynogenetic fry was demonstrated by using the mendelian segregation patterns of three recessive mutant pigment genes. Four females, heterozygous for these recessive mutations, were gynogenetically reproduced and the frequencies of mutant phenotypes in the presumably homozygous offspring was determined. The mutations concerned were blond (designated b_1 and b_2) and transparent (designated t_p) (Komen, 1990). Homozygous $b_1,b_2/b_1,b_2$ fry lack normal melanophore development and have a yellow phenotype. Heterozygous animals $b_1,+/b_1,b_2$, $+,b_2/b_1,b_2$ and $+,+/b_1,b_2$ all show normal melanophore development and cannot be distinguished from each other.

Homozygous t_p/t_p fry are completely transparent as a result of reduced guanophore development. Heterozygous animals $+/t_p$ are normal in appearance. The 3 genes b_1, b_2 and t_p are not linked to each other. Fry which are both blond and transparent lack melanophores and are translucent with black eyes. All phenotypes are clearly distinguish-

able 4 weeks after hatching (Komen, 1990).

Five replicate groups of 400- 1200 eggs from each of 3 females, heterozygous for b_1 or b_2, and t_p, were fertilized with irradiated milt and heat shocked (40.0 °C, 2 min) at 30 min after fertilization. Eggs from a fourth female, heterozygous for both b_1 and b_2, and t_p, were similarly treated. After hatching the gynogenetic fry from the 5 replicate groups of each female were counted and pooled. Fry from the controls of sperm irradiation of each female were also pooled. After 4 weeks the mortality in each group was assessed and the frequencies of blond, blond/transparent, transparent and normal pigmented fry determined.

All 4 females were homozygous for a recessive mutation in the S (scalation) gene and had a scattered phenotype (s/s: mirror carp). The (D1 x W15)-male used was heterozygous S/s and scaled. A possible genetic contribution from insufficiant irradiated sperm was assessed by the occurence of scaled fry in gynogenetic offsprings and controls for sperm irradiation.

Production of homozygous clones and F1 hybrids.

Two females (E4 and E20) and two males (E5 and E6) from one presumably homozygous gynogenetic offspring were used to produce two gynogenetic homozygous inbred strains and four F_1 hybrid groups. The offspring had been raised to maturity in the hatchery of the central fish culture facilities at the Wageningen Agricultural University as already described (see husbandry). The females used were two of the few that could be reproduced succesfully at an age of 2 years (see also discussion). The males in this broodstock were homozygous for a recessive mutation in a sex determining gene designated mas-1. They can be considered as true XX-males (Komen and Richter, 1990). Homozygous gynogenetic inbred strains (clones) were produced by cold shocking eggs from females E_4 and E_{20} to inhibit the second meiotic division as described previously (Komen et al., 1988). Eggs were fertilized with irradiated milt and shocked at 0°C for 45 minutes, 1-2 min after fertilization. F_1 hybrids were produced by fertilizing eggs from the E4 and E20 females with non-irradiated milt from the E5 and E6 males. The clonal nature of the strains was examined twenty weeks after hatching by skin grafting between individuals of the same strain (Komen et al., 1990). The fish were

arranged in pairs and one graft was reciprocally exchanged in each pair. This was carried out with 24 fish per strain with the exeption of the E_4 x E_5 and E_{20} x E_5 strain from which only 8 individuals were tested. As a control on the immuno-competence of the homozygous fish grafts were likewise exchanged between fish from the E_{20} and E_4 clones. This was done with 12 fish from each clone.

Table 1 The survival of embryos after 24 hrs and the yield of normal fry after 96 hrs (%) from eggs, heat shocked at various times after fertilization. Each trial refers to the offspring of a different male and female. Eggs were incubated at 24 °C and shocked at 40 °C for 2 minutes.

embryo	time after fertilization (min)										
trial	24	26	28	30	32	34	36	38	40	A	B
1			54.9	15.1	0.0	0.0	5.1		64.0	96.3	88.9
2	2.6	7.5	21.0	31.1	17.7	14.4	4.6			75.9	44.0
3	10.3	11.7	14.5	20.3	8.6	0.7	0.3			84.7	65.0
4		57.8	39.8	24.0	17.4	0.0				97.9	59.7
5		32.2	37.8	21.5	12.1	0.7				71.8	72.5
6			27.5	23.0	12.3	6.5	0.4	0.6	5.7	98.3	89.1
normal fry											
trial	24	26	28	30	32	34	36	38	40	A	B
1			10.6*	4.8	0.0	0.0	0.3		2.0	92.6	0.2
2	0.0	0.6	0.7	11.6*	6.6*	9.4*	0.0			49.5	0.9
3	0.0	0.6	1.4	2.4	0.3	0.0	0.0			65.9	0.0
4		1.1	5.6*	2.2	2.9	0.0				96.7	0.7
5		2.2	12.2*	5.8*	5.4	0.7				71.3	0.5
6		2.8*	5.2*	1.4	1.5	0.0	0.0	0.0	0.0	95.8	0.5

A = control of egg quality; B = control of sperm irradiation.

* = yields of normal fry (mean values of duplicates) significantly different from sperm control according to Duncan's multiple range test ($P<0.05$).

Randomly sampled fish from each strain were transferred to aquaria with recirculated and U.V. sterilized water (23 °C), and individually numbered by tattooing (Langley hypodermic, Langley, U.K.). A 3x5 mm full thickness skin allograft was taken from the dark (many melanophores) dorsal side of the donor and slipped trough an incision under the pale ventral skin of the recipient. Two days later the recipient skin covering the graft was removed. Autografts, likewise taken from the dorsal skin of the recipient and grafted next to the allograft, served as a control on transplantation technique. Grafts were examined under a low power microscope every two days until 14 days after transplantation, and once a week thereafter until full acceptance was accomplished. The extent of hemorrhaging and the degree of melanophore destruction and regeneration was recorded.

Parameters and statistical analysis.

The yields of normal fry (%) after hatching and yolk-sac absorbtion (t_{96}) in the various treatment groups and controls for sperm irradiation of the heat shock optimisation trials were compared to determine the effects of the heat shock treatments. All data were transformed using an arc-sin transformation and analysed with Duncan's multiple range test ($P < 0.05$)(Sokal and Rohlf, 1969) using an SPSS computer program (Nie et al., 1975). The yields of homozygous fry were similarly used to compare the sensitivity of the eggs from the four different females to the gynogenetic treatment. Homozygous gynogenetic progeny of the b1,+,+/b1,b2,tp females were expected to contain equal ratios of blond, transparent, blond/transparent and normal fry. Similarly the +,+,+/b1,b2,tp female (nr 4, table 3) should produce 25 % blond and blond/transparent fry in equal ratios, and 75 % normal and transparent fry in equal ratios. A chi-square test for goodness of fit ($P < 0.05$) was used to determine whether these frequencies of normal, blond, transparent and blond/transparent fry in the four groups of offspring deviated significantly from the expected ratios.

RESULTS

Histological examination of carp embryos during the first mitotic division (Figure 1).

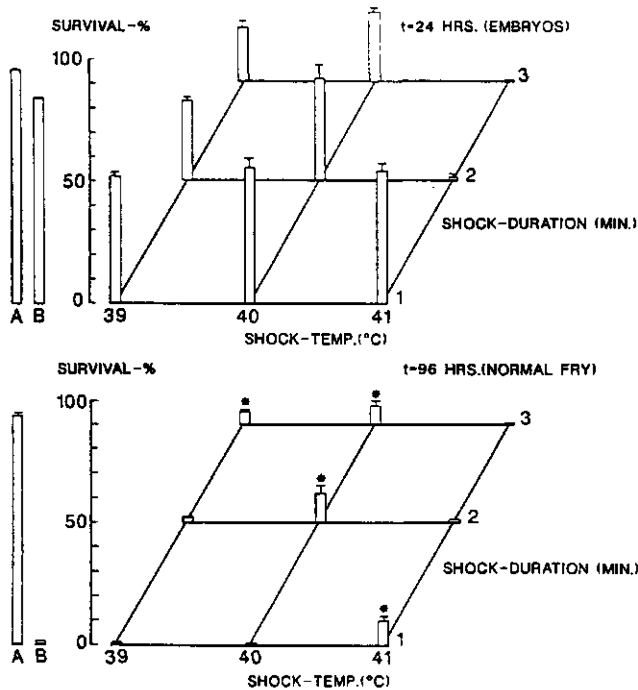
All eggs examined showed normal development, indicated by the formation of a large blastodisc at the animal pole and the migration of yolk vesicles towards the vegetal pole. The first mitosis of the embryo occurs between 20 and 35 min after fertilization (a.f.). At 20 min a.f. the male and female pronuclei fuse to form a large nucleus with associated asters. The first mitosis is initiated at 25 min a.f. by a condensation of the chromosomes and the formation of spindle poles. At 28 min a.f. the embryo enters prometaphase: the nuclear envelope dissolves and the chromosomes attach themselves to the spindle fibers. During metaphase between 30 and 32 min a.f. the chromosomes are aligned across the middle of a conspicuous spindle. The sister-chromatids are separated during the following ana/telophase and pulled towards the spindle poles. The asters increase in size and at 40 min a.f. a cleavage furrow can be seen. The chromosomes decondensate and form two new daughter nuclei.

Heat shock treatments (Table 1; Figure 2)

High rates of developing embryos and yields (49.5 - 96.7 %) of normal fry were found in the controls for milt and egg quality. All controls for sperm irradiation had similar high rates of developing embryos but only few normal fry (0 - 0.9 %) were recovered, indicating complete genetic inactivation of sperm without loss of fertilization capacity. The survival of embryos and yields of normal fry of eggs shocked at different times after fertilization (a.f.) were found to correlate with the successive stages of mitosis (compare figure 1 and table 1).

The survival of embryos increased progressively with eggs shocked at t24, t26, t28 and t30 a.f., i.e. from prophase to prometaphase, and decreased when eggs were shocked at later stages of the mitotic division. There was some variation between the trials concerning the location of the optimum survival range. Survival was usually highest (20.3-57.8 %) when eggs were shocked between 26 and 30 min a.f.

Figure 2 The survival rate of embryos and yields of normal fry (%) from eggs, shocked at different combinations of heat shock temperature and duration. Eggs and sperm originate from the same female and male. Eggs were incubated at 24 °C and shocked 30 minutes after fertilization.



A = control of egg quality; B = control of sperm irradiation
* = yields of normal fry (mean values of duplicates) significantly different from the sperm irradiation control according to Duncan's multiple range test ($P < 0.05$).

Statistically significant yields of normal fry (5.2 - 12.2 %, table 1) were obtained when eggs were shocked at 28-30, and occasionally 32 minutes a.f. Earlier or later shocks resulted in few surviving fry, not significantly different from the control of sperm irradiation. Varying the heat shock temperature and duration revealed a narrow range of permissive shock combinations (Fig.2). Between 25.4 and 56.9 % of the eggs shocked at 39 °C for 3 min, at 40°C for 2 and 3 min, or at 41°C for 1 min, developed into

Table 2 The survival of embryos after 24 hrs, yields of normal fry after 96 hrs and mortality of fry between hatching and 28 days post hatching from eggs of 4 different females, heterozygous for "blond" and "transparent". Eggs were incubated at 24 °C and shocked (40.0 °C, 2 min) 30 minutes after fertilization.

Female	replicate	Number of eggs	survival t_{24} embryos (%)	survival t_{96} normal fry (%)	normal fry*) (mean ± sd)	mortality %
1	1	1022	67.3	10.1		
	2	790	80.3	12.3		
	3	1068	60.7	9.8	8.6 ± 2.9	57.3
	4	742	66.2	7.5		
	5	1294	51.6	4.8		
	B	912	89.1	0.6		
2**	1	947	69.1	15.7		
	2	1676	57.3	3.5	8.8 ± 5.1	48.0
	3	878	64.1	8.9		
	4	861	65.9	6.9		
	B	839	82.8	0.8		
3	1	494	62.6	14.4		
	2	748	61.9	12.6		
	3	1014	59.8	10.1	11.3 ± 2.3	44.4
	4	1139	58.9	10.1		
	5	771	66.3	11.3		
	B	938	91.0	0.7		
4	1	557	47.2	11.0		
	2	455	55.0	9.2		
	3	610	53.9	6.8	9.3 ± 2.3	36.1
	4	782	48.7	7.2		
	5	389	53.0	12.3		
	B	887	93.4	0.9		

* control for sperm irradiation (B) not included

** one replicate was excluded due to fungus infection.

normal embryos after 24 hrs and produced statistically significant yields of normal fry (4.8-14.3 %). Eggs shocked at 39°C but for shorter durations showed a similar survival of embryos but did not yield significant numbers of normal fry. Eggs, shocked at 41°C for 2 or 3 minutes exhibited nearly 100 % mortality within 24 hrs.

Table 3 Frequencies for normal, transparent, blond and blond/transparent fry in presumed homozygous gynogenetic offspring from 4 different females, 28 days after hatching (see also table 2).

Female	genotype[1)]	day 20	normal		transparent		blond		blond/transp		Chi^2
		N	N	%	N	%	N	%	N	%	
1	$(+,b_2,+/b_1,b_2,t_p)$	180	44	24.4	43	23.9	48	26.7	45	25.0	0.31
2	$(+,b_2,+/b_1,b_2,t_p)$	179	43	24.0	44	24.6	47	26.3	45	25.1	0.20
3	$(+,b_2,+/b_1,b_2,t_p)$	261	58	22.2	57	21.8	74	28.4	72	27.6	3.72
4	$(+,+,+/b_1,b_2,t_p)$	159	55	37.7	60	34.6	20	12.6	24	15.1	1.22
B		20	11	55.0	7	35.0	0	0	2	10.0	12.8*

1 the genotypes $(+,b_2,+/b_1,b_2,t_p)$ and $(b_1,+,+/b_1,b_2,t_p)$ cannot be distinguished
B control for sperm irradiation (pooled values)
* the observed frequencies for normal, transparent, blond and blond/transparent fry are significantly different from the expected frequencies according to the Chi-square test for goodness of fit ($P_{0.05}$ = 7.82).

Incidence of homozygosity in gynogenetic fry (table 2).

All 4 females produced relatively high numbers of gynogenetic fry. Differences in yields of normal gynogenetic fry between females were insignificant but there was a considerable variation in yields between replicate groups of each female (3.5 -15.7% for female nr 2). All offspring suffered considerable mortalities (36.1 - 57.4 %) during the first 28 days after hatching. The observed frequencies of normal, blond, transparent and blond/transparent fry in each group of pooled offspring from the 4 females after 4 weeks were not significantly different from the expected frequencies (table 3). There was a slight excess of blond and blond/transparent fry in the offspring of female nr 3. Only 20 normal fry from the pooled sperm irradiation control survived until 4 weeks after hatching. Eleven were normally pigmented, 7 were transparent and two were blond/transparent. This ratio was significantly different from either a 1:1:1:1 or 3:3:1:1 ratio ($p<0.05$). None of the offspring, including the sperm irradiation control, contained scaled fry indicating a complete absence of paternally inherited genes.

Table 4 Skin grafting between individuals of presumed homozyous clones and F1 hybrids.

Strain	Number of fish	allografts			autografts	
		accepted	lost	rejected	accepted	lost
E4 gyn	24	21	3	0	22	2
E20 gyn	24	22	2	0	23	1
E4 x E6	24	20	4	0	21	3
E20 x E6	24	18	6	0	19	5
E4 x E5	8	8	0	0	8	0
E20 x E5	8	6	2	0	7	1
E4 vs E20	12 vs 12	0	2	22	22	2

Homozygous gynogenetic clones were produced by cold shocking eggs from females E_4 and E_{20} at $0^{\circ}C$, 1-2 minutes after fertilization. F_1 hybrids were produced by fertilizing eggs from the E4 and E20 females with non-irradiated milt from the E5 and E6 males. For skin grafting the fish were divided into pairs and one allograft was reciprocally exchanged per pair. Grafts were also reciprocally exchanged between 12 fish from the E4-gyn and 12 fish from the E20-gyn groups.

Homozygous clones and F1 hybrids (Table 4).

The clonal nature of the homozygous inbred and F1 strains was confirmed by the permanent acceptance of allografts. Some allografts failed to adhere to the host and were lost after removal of the recipient skin. Allografts reacted identically to the autografts with respect to damage and regeneration of pigment and the extent of hemorrhage. All grafts showed some vascularisation which disappeared between 6 and 8 days after transplantation. Destruction of pigment cells, due to mechanical damage, was observed during the first 6 days after transplantation. Regeneration of melanophores started around 10 days after transplantation, and after 3 weeks the graft was completely

healed and accepted. Grafts exchanged between E_{20} and E_4 fish were rejected within 12 to 16 days after transplantation. All allografts showed extensive vascularisation and hemorraghing. Melanophore destruction started 4 days after transplantation and was completed during the following 8 - 12 days.

DISCUSSION

In our experiments consistent yields of 5 -12 % (max 15.7) viable homozygous fry were produced when haploid eggs, incubated at 24°C, were subjected to a heat shock of 40°C for 2 min, 30 min after fertilization, i.e. at metaphase of the first mitotic division. Similar conditions of the heat shock and time of application were reported by Nagy (1987) who, in a single experiment, produced gynogenetic fry with a heat shock applied at 40 min after fertilization. This difference can be explained by the different incubation temperatures used (24°C vs 22 °C), but more likely has a genetic basis (Komen et al.,1988). Comparable differences in embryonic development rate are found between various inbred mice strains (Hansmann et al., 1985).

Heat, cold and pressure shocks are known to be effective in disrupting the microtubili that make up the spindle during mitosis and meiosis (Dustin, 1984). Applied at metaphase, they cause failure of the mitosis and subsequent cellular division (Reinschmidt et al., 1979; Streisinger et al., 1981; Onozato, 1984). The productive heat shock combinations (39°C,3 min to 41 °C,1 min) found in this study are a little higher than those reported by Hollebecq et al. (1986) but might be typical for most Cyprinids. Heat shocks of 41°C for 2-3 min are lethal in common carp but succesfully induced diploidy in *Oreochromis niloticus* (Mair et al.,1987), *Brachydanio rerio* (Streisinger et al.,1981), and *Oryzias latipes* (Naruse et al., 1985). For Salmonids, optimum heat shock temperatures of 36-38 °C have been reported (Thorgaard et al., 1981).

There is a remarkable consensus regarding the low and variable yields of homozygous fry reported in all these studies, irrespective of whether androgenesis or gynogenesis is applied (Chourrout, 1987). Partly this appears to be a consequence of the critical conditions of the shock treatment (see fig 2 and table 2). However, the fraction of eggs in metaphase at the time of shock treatment will determine the fraction of eggs that can

respond favourably to the shock. Common carp eggs, cold shocked shortly after fertilization, are all in metaphase II of the meiosis and yields of normal fry are usually high (25-50 %: Nagy et al., 1978; Komen et al., 1988). The variable and increased range of productive shock times around 30 minutes after fertilization (see table 1) indicate some variation in developmental rate between eggs of the same spawn. Synchronously developing eggs, in conjunction with proper timing of the first mitosis, are therefore imperative for succesfull gynogenesis (Streisinger et al., 1981; Chourrout, 1984; Naruse et al., 1985).

An important problem associated with very low yields of gynogenetic fry is the contamination with heterozygous spontaneous diploids which occasionally survive suboptimal shock treatments (Purdom et al., 1985; Linhart et al., 1987). Spontaneous diploids arise from non-disjunction during the first meiotic (M-I) or second meiotic (M-II) division and this mechanism might be genetically controlled, as was demonstrated in common carp (Cherfas, 1981), plaice (Thompson et al., 1981) and mice (Bartels et al., 1985). In this study we used two pigment markers to detect M-I or M-II nondisjunction. M-I non-disjunction results in 100 % heterozygous offspring for genes located close to the centromere, such as transparent. Similarly, M-II nondisjunction in gynogenetic offspring from $+,+/b_1,b_2$ females is detected by the presence of approximately 6 % blond fry (Komen, 1990). However, we did not find evidence for the existence of any substantial numbers of heterozygous fry in the presumed homozygous offspring (see table 3). On the other hand, the presence of nearly equal numbers of transparent and normal fry, and the almost absence of blond fry in the pooled control for sperm irradiation (table 3) suggest an origin from non-disjunction in meiosis II for these fry. It is unknown whether these diploids actually survive optimal heat shock treatments, since this should result in tetraploid fry. Experiments designed to produce tetraploid carp were unsuccesful (unpublished results) while screening of large numbers of homozygous gynogenetic fry has never revealed the presence of anything but diploids. However, it cannot be excluded that very few heterozygous fry do survive the treatment but are outnumbered by high yields of homozygous gynogenetic fry, as was suggested by Purdom (1985). Rigorous screening of large numbers of presumably homozygous fish for electrophoretically detectable heterozygous phenotypes of various enzymes could

resolve this question.

The ultimate proof for homozygosity came from transplantation testing of the homozygous clones and F_1 hybrids, produced from the presumably homozygous broodstock. Skin (scale) grafting has been succesfully used to demonstrate the clonal nature of natural occurring gynogenetic populations of Xiphophorus maculatus (Kallman, 1970) and triploid clonal Carassius gibelio langsdorfii (Nakanishi et al., 1987). However, in the only two reports on gynogenetic homozygous clones of fish (Streisinger et al., 1981; Naruse et al., 1985) this conclusive piece of evidence was omitted.

In carp, histo-compatibility genes exist as at least one major locus and multiple minor loci, which are codominantly expressed. Even small differences in minor histocompatiblity genes result in chronic rejections of grafts (Komen et al., 1990). However, all allografts exchanged among members of the homozygous clones or F_1 hybrids were unequivocally accepted. Furthermore grafts exchanged between homozygous E_{20} and E_4 fish were all rejected (table 4). These results illustrate the power of this technique for future screening of clones of common carp and other fish species.

Homozygous clones and their hybrids will open up new exciting areas for selective breeding. The hybrid strains show an important reduction in variation for various morphological traits when compared to an outbred strain (Komen et al., in prep). Since most variability in common carp is probably non-additive (Moav and Wohlfarth, 1968), selected hybrids are expected to show important heterosis as well, as has been outlined by Cherfas (1981), and Nagy (1987). However, the production of homozygous clones is not without problems. The inbreeding depression expressed in homozygous gynogenetic offspring is considerable and many fish are lost during weaning (see table 2) and maturation (25 - 35 %). Furthermore, many mature homozygous females show severe defects in their gonads and only a few (< 10 %!) can be gynogenetically reproduced (Komen et al., in prep.). These problems might illustrate why only three fish species have been cloned so far, despite numerous reports on the production of homozygous fry (*Brachydanio rerio*: Streisinger et al., 1981; *Oryzias latipes*: Naruse et al., 1985; *Cyprinus carpio*: Komen et al., this paper).

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Chapter 5

SKIN GRAFTING IN GYNOGENETIC COMMON CARP (*Cyprinus carpio*, L.) THE DEVELOPMENT OF HISTOCOMPATIBLE CLONES.

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ABSTRACT

The fate of skin allografts exchanged among heterozygous and homozygous gynogenetic common carp siblings, and among newly developed inbred strains and F_1 hybrids , is described. Heterozygous gynogenetic offspring were produced by fertilizing eggs with U.V.- irradiated sperm and by treating the resulting zygote with a cold shock ($0^{\circ}C$, 45 min). The temperature shock causes retention of the second polar body which allows the eggs to develop into normal diploid fry. Homozygous gynogenetic offspring were similarly produced by using a heat shock ($40^{\circ}C$, 2 min) which suppresses the first mitotic division. Skin allografts exchanged among heterozygous gynogenetic carp exhibited prolonged survival with some allografts (21.8 %) surviving for over 28 days. Furthermore a strong histocompatibility locus was seen to seggregate in this group. In contrast skin allografts exchanged among homozygous gynogenetic siblings were all rejected within 14 days (MST 9.4 days). New homozygous inbred strains, designated JJ and MM, were produced by gynogenetic reproduction of homozygous female carps, while F_1 hybrids were produced by crossing of these homozygous females with homozygous male siblings. All grafts exchanged among members of the same strain were permanently accepted. Likewise grafts from homozygous strain members were accepted by fish from the related F_1-hybrids, while the reverse grafts were rejected. These results provide evidence for the idea that in carp histocompatibility genes exist as at least one major locus and multiple minor loci, which are codominantly expressed.

INTRODUCTION

In all mammals, birds and amphibians studied, a group of closely linked genes, called the major histocompatibility complex (MHC), controls the production of a strong transplantation antigen that elicits acute allograft rejections (1, 2). Other, minor histocompatibility loci code for antigens which produce variable, but usually weak immune responses (3). Little is known about the organization of histocompatibility genes in fishes. Most teleosts consistently reject allografts in an acute fashion (4,5,6) but estimates on the number of histocompatibility loci involved vary from 4-7 in goldfish (7) to 10-15 in different *Xiphophorus* species (8). Furthermore, reactions against allo-antigens in vitro, as demonstrated by mixed leucocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) also appear to be regulated by more than one histocompatibility locus (9).

The presence of a well developed immune system in carp (*Cyprinus carpio*) argues for the existence of a MHC homologue in these species (10,11). However, studies on skin-allograft reactions or MLR using full-sib and gynogenetic offspring of common carp have been inconclusive (12-15). Comparable studies in the amphibians *Xenopus laevis* (16) and *Rana pipiens* (17) demonstrated a single strong histocompatibility locus responsible for acute graft rejection.

More affirmative evidence for the existence of a MHC homologue in carp could be obtained by using inbred strains and F_1 hybrids . Such strains can be produced by gynogenesis. In gynogenesis, eggs from common carp are fertilized with sperm which is genetically inactivated by U.V.-irradiation. Diploidy is restored by suppression of the second meiotic or first mitotic division. In the first case the resulting offspring are partly homozygous and heterozygous for those genes involved in recombination events between non-sister chromatids during the first meiotic division (heterozygous gynogenesis) (18, 19). In the second case the post-meiotic haploid maternal genome is duplicated before the first mitotic division. Suppression of this division therefore results in fully homozygous diploid offspring (homozygous gynogenesis)(20, 21). Subsequent gynogenetic reproduction of homozygous fish will yield fully homozygous (inbred) strains while crosses between homozygous siblings result in strains of genetically identical but partly heterozygous fish (F_1 hybrids). In the present paper, the two modes of gynogenetic reproduction were used to investigate the segregation of histocompatibility genes in the offspring, as inferred from the fate of skin allografts. Random selected homozygous carps were then propagated to produce homozygous inbred strains and F_1 hybrids . The clonal nature of the strains was studied by intra- and inter-strain graft exchange. Knowledge on the organization of histocompatibility genes in carp will facilitate the production of inbred strains with particular histocompatible genotypes. Such strains will be extremely useful for basic studies on the genetics of the immune response and the existence of a MHC at the evolutionary level of fish.

MATERIALS AND METHODS

Animals. Broodstock of common carp (*Cyprinus carpio L.*) were derived from a cross D1(♂) x W15(♀). D and W stands for random-bred German (D) and Dutch (W) strains. From this broodstock, one male and one female were selected for reproduction. Eggs

and milt were obtained by artificially induced reproduction as described before (19). Fry were raised in aquaria with recirculated and filtered water (24°C). They were fed *Artemia salina* nauplii for the first two weeks after hatching, followed by trout-pellets according to the recommendations of the manufacturer (Trouw, Putten, the Netherlands).

Gynogenesis.

Gynogenesis was induced as described previously (19, 21). Genetic inactivation of sperm was achieved by irradiating milt with U.V.(2200 J/m2/min) for 60 min. Heterozygous gynogenetic fry were produced by treating eggs, fertilized with irradiated milt and incubated at 24 °C, with a cold shock (0°C, 45 min) starting 7-9 min after fertilization. The cold shock causes retention of the second polar body (2PB-group). Approximately 35 % of the eggs developed into normal, diploid fry. To produce homozygous gynogenetic fry, eggs were fertilized with irradiated milt, incubated at 24°C, and heat shocked (40°C, 2 min.) 30 min after fertilization. The heat shock causes endomitosis in the absence of a first cellular division (EM-group). Only 11% of the treated eggs developed into viable diploid fry. Control, full-sib fry were obtained by fertilizing eggs with non-irradiated milt (CO-group). Typically, more than 90 % of these normal fertilized eggs developed into fry.

Inbred strains and F_1 hybrids

Two females (J and M) were randomly chosen from the EM-group and used for the production of homozygous inbred lines. Eggs from each female were fertilized with irradiated milt from an unrelated male and incubated at 24°C. They were then cold shocked (0°C, 45 min) 7-9 min after fertilization to produce two different groups of genetically identical homozygous fry (inbred strains JJ and MM). Two groups of genetically identical but partly heterozygous fry were produced by fertilizing the eggs from each female with milt from a homozygous male (S) of the EM-group (F_1 hybrids JS and MS).

Genetic markers.

The original (WxD)-female used in this experiment was homozygous for a recessive gene inhibiting normal scalation (mirror carp: s/s) and heterozygous for two recessive mutants

(designated b1 and b2) inhibiting melanophore development (black: b_1,+/+,b2). The (WxD)-males used were heterozygous scaled (+/s) and homozygous black (+,+/+,+). The absence of scaled fry in presumptive gynogenetic groups provides a reliable check for the absence of paternal genes. The degree of homozygosity in 2PB- and EM-groups was estimated by the number of blond fish (no melanophores: b1,b2/b1,b2). In heterozygous gynogenetic fry (2PB-group), due to a high cross over rate, only 3-6% blond fish are normally found, while in homozygous gynogenetic fish approximately 25% are blond (21).

Experimental design.

In a first series of experiments, grafts were reciprocally exchanged within groups of 5 fish, which were randomly chosen from the CO-, 2PB- or EM-group. Each fish in a group received 4 allografts and 1 autograft. Three groups of 5 CO fish, 4 groups of 5 2PB fish and 4 groups of 5 EM fish were tested. The fish were 6 months old at the start of the experiments (mean weight 150 g).

In a second series of experiments, grafts were reciprocally exchanged between a group of 5 2PB fish and a group of 5 EM fish. Each fish received 1 allograft from each fish in the other group, and 1 autograft. The fish were 9 months old at the start of the experiment (mean weight 300 g).

In a third series of experiments, grafts were reciprocally exchanged between fish from the inbred strains and F_1 hybrids. A fish from strain JJ received 1 allograft from another individual of strain JJ, and one from a fish belonging to strain MM and the hybrid group JS. Similarly, an animal from strain MM received an allograft from fish of strains MM, JJ and hybrid group MS. Fish from the MS hybrid group received grafts from individuals of the hybrid groups MS and JS and from strain MM, and fish from the JS group received allografts from donors of hybrid groups JS and MS, and strain JJ. In this way, 16 fish of each strain or hybrid group were tested. The fish were 6 months old at the start of the experiments (mean weight 250 g).

Transplantations.

Fish were transferred to aquaria with U.V.-sterilized water of 22°C and individually numbered by tattooing (Langley, hypodermic, U.K.). Skin grafting was started after an adaptation period of 2 weeks. Only mirror carp females with normal melanophore

development were used. Donor and recipient were anaesthetised in 0.03% MS 222 (Sandoz) in water. Grafting was performed under a low power stereomicroscope. A 5 mm incision was made in the pale (few melanophores) ventral skin of the recipient. A 3x5 mm, full-thickness skin allograft was taken from the dark (many melanophores) dorsal side of the donor, and slipped through the incision under the recipient skin. Two days later the overlying recipient skin was removed from the graft. Autografts were taken from the dorsal side of the recipient and transplanted to the ventral side of the same animal. Grafts were examined every other day and the fraction (%) of intact melanophores in the graft was estimated. The survival end point of the graft was defined as the number of days after transplantation at which all melanophores were destroyed. After day 28, regeneration of the underlying and surrounding recipient skin made a distinction between recipient and donor melanophores difficult and the examination of allografts was stopped. Only in the third series of experiments (see experimental design) graft examination was continued after 28 days with intervals of 5 days until a graft was completely destroyed. The median survival time in the first and second series of experiments was calculated as the day after transplantation when 50 % of the allografts in the experimental groups were rejected. In the third series of experiments, the mean survival time ± sd was calculated, since all allograft reactions in one group are in fact replicates.

RESULTS

The gynogenetic groups did not contain scaled individuals, indicating the absence of paternal genes in these fry. Fish from the CO-group (n = 292) were either scaled (51.4%) or scattered (48.6%) as expected. The number of blond fish in the EM-group (45 out of 211) and 2PB-group (12 out of 298) fitted the expected percentages of 25 % and 3-6 % respectively. No blond fish were found in the CO-group.

First series of experiments (table 1).

The median survival time of allografts in fish from the CO-group was 10.4 days. Autografts and allografts are indistinguishable for the first 2 days after transplantation. Blood recirculation is restored and the grafts become slightly inflamed. Mechanical damage to the grafts is manifested as local areas of destructed melanophores (less than

10 %). Autografts eventually heal in well and can not be distinguished from the surrounding host tissue after 12 days. Allografts become covered by hyperplastic host tissue and show vascularisation and hemorrhaging after 4 days. Melanophore destruction usually begins after 6-10 days and is completed within 8-18 days after transplantation.

Table 1 Survival end points of allografts, exchanged within groups of 5 fish from different experimental groups (FS, 2PB or EM group).

	Days after grafting										
	8	10	12	14	16	18	20	22	24	26	28
CO group											
exp. 1		6	6	3	1						
2	4	6	5								
3		6	5	2	3	3					
total	4	18	16	5	4	3					
percentage	8.0	36.0	32.0	10.0	8.0	6.0					
								MST 10.4 days			
EM group											
exp. 1	5	7	6	1							
2	4	4	4								
3		10	5	3							
4	2	10	5	1							
total	11	31	20	5							
percentage	16.4	46.3	29.9	7.4							
								MST 9.4 days			
2PB group											
exp. 1	1	2	5					1	1	1	1
2		1	3	2	2	2	1	2	2		1
3			3	1	2	2	3		2		5
4			2	5	1	1	1	2			7
total	1	3	13	8	5	5	4	5	5	1	14
percentage	1.6	4.7	20.3	12.5	7.8	7.8	6.3	7.8	7.8	1.6	21.8
								MST 16.8 days			

Fish from the EM-group rejected all allografts within 14 days after transplantation (MST 9.4 days), whereas all autografts were accepted. Allograft-reactions were vigorous with both graft and surrounding host tissue showing extensive vascularisation and hemorrhaging. Observation of the graft was often hampered by dense hyperplastic host tissue

Table 2 Survival end points of allografts, exchanged within groups of 5 heterozygous gynogenetic fish (2PB group), classified as acute (+) or subacute/chronic (-) allograft reactions; and genotype assignments for a putative strong H-locus.

Acceptor	Donor (fish nr) (01)	(02)	(03)	(04)	(05)
(01) AA		L[1]	20 –	22 –	12 +
(02) AB	24 –		L	24 –	28 –
(03) AA	L	L		18 –	10 +
(04) AA	20 –	14 +	24 –		16 +
(05) BB	12 +	12 +	14 +	14 +	

Acceptor	Donor (fish nr) (06)	(07)	(08)	(09)	(10)
(06) AA		28 –	20 +	28 –	28 –
(07) ??	18 +		14 +	14 +	12 +
(08) BB	14 +	L		14 +	12 +
(09) AA	28 –	28 –	14 +		22 –
(10) AA	28 –	22 –	16 +	28 –	

Acceptor	Donor (11)	(12)	(13)	(14)	(15)
(11) AA		24 –	12 +	14 +	L
(12) AA	28 –		20 +	L	28 –
(13) BB	16 +	16 +		18 +	18 +
(14) AB	20 –	24 –	20 –		28 –
(15) AA	28 –	28 –	12 +	12 +	

Acceptor	Donor (16)	(17)	(18)	(19)
(16) AA		10 +	22 –	8 +
(17) BB	12 +		12 +	24 –
(18) AA	26 –	12 +		10 +
(19) BB	12 +	28 –	12 +	

1) L = grafts lost within 4 days after transplantation

covering the graft. Melanophore destruction started after 6 days and was usually completed at day 12 after transplantation. In contrast to fish from the CO or EM group, fish from the 2PB group exhibited prolonged survival of allografts (MST 16.8 days). Large differences were observed between individual allografts on the same recipient. Based on the intensity of the allograft reaction and pattern of melanophore destruction, two types of allograft reactions could be distinguished. Acute allograft reactions resembled allograft reactions in control fish. Melanophore destruction started 6-10 days after transplantation and was usually completed during the following 4-6 days. Sub-acute/chronic allograft reactions showed markedly less vascularisation and hemorrhaging than acute allograft reactions. Melanophore destruction started late, 10-14 days after transplantation, and proceeded in an uneven way during the following 8-14 days. Some parts of the graft were destructed earlier than others. In some recipients all allograft reactions were markedly delayed. In these fish, chronic allograft reactions were very weak with allografts surviving for over 28 days without visible melanophore destruction. Classifying each individual allograft reaction as acute (+) or subacute/chronic (-), all 5 fish in each experiment were assigned a putative genotype for a strong histocompatibility locus with alleles A and B (table 2). It is assumed that allografts from donors with the same genotype are chronically rejected, while grafts differing in one or both alleles from the recipient are rejected in an acute fashion. Fish with a heterozygous genotype (AB) reject all allografts chronically.

Second series of experiments (table 3).

More than 90 % of the allografts from 2PB fish were rejected within 12 days after transfer to EM fish (MST 9.4 days). Allograft reactions resembled those observed in grafts, exchanged among fish from the EM-group (see table 1). In contrast, skins from EM fish, grafted on 2PB fish, exhibited prolonged survival (MST 11.8 days). Allograft reactions were similar to those observed in grafts exchanged among heterozygous gynogenetic fish (table 1), although no clear distinction could be made in acute or chronic allograft reactions. Six allografts survived for 26 days or longer, showing little or no melanophore breakdown.

Table 3 Survival end points of allografts, exchanged between fish from the EM group and 2PB group.

	Days after grafting										
	8	10	12	14	16	18	20	22	24	26	28
2PB on EM											
exp. 1	1	15	7	1							
2		6	4								
3	2	7	2	1	1	1					
total	3	28	13	2	1	1					
percentage	6.2	58.3	27.1	4.2	2.1	2.1					
									MST 9.4 days		
EM on 2PB											
exp. 1	1	12	3	2			1				2
2		3	6	6	2		1			2	
3			3	2	5	1	1			1	1
total	1	15	12	10	7	1	3			3	3
percentage	1.8	27.3	21.8	18.2	12.7	1.7	5.5	0	0	5.5	5.5
									MST 11.8 days		

Third series of experiments (table 4).

All allografts, exchanged among fish of the same strain, were permanently accepted. Likewise, grafts from fish of strains JJ and MM were accepted by fish of hybrid groups JS and MS (Fig. 1a-1c), while the reverse grafts were uniformly rejected (20.2 ± 2.9 days and 18.9 ± 3.8 days respectively). Allograft reactions were subacute/chronic as in 2PB fish (first experiment) and showed little variation between individual fish from the same strain. Grafts, exchanged reciprocally between fish of strains JJ and MM, were all rejected in an acute fashion which resembled allograft reactions observed in EM fish (see first experiment). Grafts from fish of strain JJ survived slightly longer on fish of strain MM (16.6 ± 3.3 days) than the reverse grafts (13.1 ± 3.3 days) but this difference was not significant. A similar but more pronounced phenomenon was seen in grafts reciprocally exchanged between fish of the JS and MS hybrid groups. Grafts from MS fish were all rejected within 30 days (25.6 ± 4.6 days) by JS fish while the reverse grafts

were rejected in a truly chronic fashion (44.8 ± 6.2 days). In these grafts, pigment destruction started after 40 days, with a low degree of haemorrhaging.

Table 4 Mean survival endpoints of allografts exchanged between fish of inbred (KK and MM) and recombinant (KS and MS) strains.

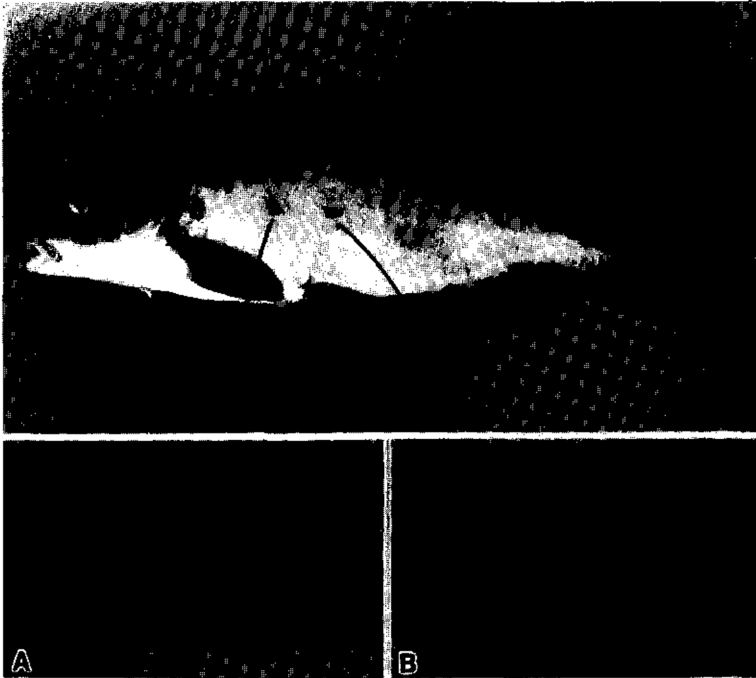
ACCEPTOR STRAIN	DONOR STRAIN: KK	MM	KS	MS
KK	accepted n = 13	13.1 ± 3.3 n = 9	20.2 ± 2.9 n = 13	X[1]
MM	16.6 ± 3.3 n = 13	accepted n = 16	X	18.1 ± 3.8 n = 15
KS	accepted n = 12	X	accepted n = 13	25.6 ± 4.6 n = 12
MS	X	accepted n = 13	44.8 ± 6.2 n = 11	accepted n = 11

1) X = not tested

DISCUSSION

The present study showed that different modes of gynogenetic reproduction result in different types of allograft rejections. In full-sib progeny (CO-group), most grafts were rejected in an acute fashion with only few grafts showing prolonged survival (> 18 days). These results are in good agreement with those of Hildemann and Owen (7), and indicate that more than one histocompatiblity locus (H-locus) is involved in graft rejection. This was confirmed by the allograft reactions observed among homozygous gynogenetic offspring (EM-group). In the case of a single strong H-locus, 50 % of the grafts from fish of this group are expected to have both alleles in common with the recipient.

Figure 1 Carp from the F1 hybrid KS group with fuly accepted grafts of a syngeneic KS donor (A) and an allogeneic KK doner (B), 120 days after transplantation.



The fact that none of these grafts survived for more than 14 days can be taken as proof that a large number of H-loci are differing between donor and recipient.

Surprisingly, grafts exchanged between fish from the 2PB group not only showed extended survival but also indicated the segregation of a strong histocompatibility locus in this group. Classifying each individual allograft reaction as acute (+) or subacute/-chronic (-), all fish exept one (nr.7: see table 3) could be divided in AA, BB and AB genotypes for a strong locus. A prolonged survival of allografts was also found by Nagy et al.(12) and Van Muiswinkel et al. (15) in gynogenetic common carp, and by Nakanishi (14) in gynogenetic goldfish, and can be explained by assuming a relatively high degree of residual heterozygosity for minor H-loci in gynogenetic offspring, as suggested by Nagy (22). The relatively small size of fish chromosomes often allows no more than a

single obligate chiasma during meiosis (23, 24). Consequently, for distal genes the frequency of recombination and thus the fraction of heterozygous gynogenetic offspring (Y), is often high. In carp this is illustrated by a number of genes with y values of more than 0.67 (25) and by the fact that histocompatible lines of carps can only be obtained after at least 4 successive generations of heterozygous gynogenesis (12). It should be stressed that the actual survival times of the grafts are the result of the discriminatory capacity of the recipient (26). Fish which are heterozygous (and thus identical) for many minor H-loci show a reduction in histo-incompatibility with minor H-loci of the graft and are more likely to show the effects of a strong locus disparity than fish which are at large homozygous for these minor loci. In this respect, the genetics of histocompatibility in common carp are identical to those in the toad *Xenopus laevis* (16) and the frog *Rana pipiens* (17). In both species the presence of a strong MHC locus was demonstrated in a group of heterozygous gynogenetic offspring with reduced histo-incompatibility for minor H-genes.

It was expected that the effects of a strong H-locus disparity on graft rejection would also show when homozygous (EM) skins were grafted on their heterozygous (2PB) siblings. However, the majority of the grafts, 81.8 %, were rejected within 16 days without evidence for a strong locus effect (compare table 1 and 3). The most likely explanation is that minor H-loci are in fact more antigenic in homozygous than in heterozygous form (3). Homozygous (EM) grafts are only rejected because both alleles of a given H-locus are different from the recipient, regardless wether this is a 2PB or EM fish. In contrast, a large number of heterozygous (2PB) grafts are rejected because only one allele differs from the recipient (EM or 2PB). This allelic dosage effect apparently overrides the higher probabilities for homozygous grafts to be accepted. Similar findings have been obtained by Kallman (27) in inbred strains and F_1 hybrids of the swordtail *Xiphophorus maculatus*.

The homozygous constitution of fish from the EM group was unequivocally demonstrated by the permanent acceptance of syngeneic grafts in the homozygous JJ and MM strains and the heterozygous MS and JS strains produced from these fish. Furthermore all JJ and MM grafts were accepted by JS and MS fish while the reverse grafts, which had one haploid genotype in common with their recipient, were rejected in a chronic fashion (see table 4). Finally, all allografts reciprocally exchanged between members of the two inbred strains were clearly rejected. Skin transplantation in common

carp was thus found to conform to the basic laws of transplantation (28) and showed that H-loci are indeed codominantly expressed in this species.

The considerable discrepancy in rejection times of JS and MS grafts on MS and JS fish (44.8 vs. 25.6 days, table 4) indicate again a strong H-locus disparity between the two strains. The fish from strain JJ should be AA and fish from strain MM BB if we assume strain MS to be AB for a single strong H-locus, and fish from strain JS AA. The rejection of MS grafts by MM fish should then be more acute than the rejection of JS grafts by JJ fish. However, the observed rejection times in these groups (20.2 and 18.9: see table 4) do not fully support such a conclusion. One possible explanation is that the presumed strong H-locus is polymorphic and exists in allelic forms of different strength, as shown in mice and man (28). It can not be excluded however that the MM strain is in fact a low responding strain, due to certain defects in the immune system. This would also explain why JJ grafts are rejected slower than expected by MM fish.

The experimental animals used in this study were hybrids of two selectively bred carp strains from distinct geographic areas, Dutch and German. This is probably the reason why the effects of a strong locus were so readily noted while in other studies, using gynogenetic fish from a single strain, this was not the case (12, 14, 15). The notion that this strong histocompatibility locus is in fact a homologue of the major histocompatibility complex in mammals and birds remains an intriguing possibility. Recently, a H locus with MHC class I-like characteristics has been identified using allo-antisera raised in another gynogenetic offspring of common carp, kept at our laboratory (29). It will be interesting to see wether such antisera can be used to identify the strong H-locus alleles in the inbred strains, discussed in this paper. Eventually, the demonstration of genetic linkage between these serologic specificities, acute graft rejection, MLR and GvH reaction will permit this histocompatibility system to be functionally defined as MHC (2).

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Chapter 6

THE EFFECTS OF HETEROZYGOUS AND HOMOZYGOUS GYNOGENETIC INBREEDING ON SEX, GONAD DEVELOPMENT AND FERTILITY IN COMMON CARP (Cyprinus carpio L.)

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ABSTRACT

The gonad development and fertility in three offsprings, produced by full sib breeding (FS group: F = 0.25), heterozygous gynogenesis (2PB group: F = 0.65) and homozygous gynogenesis (EM group: F = 1), using the same common carp female, is compared. Heterozygous gynogenetic offspring were all female, but the homozygous offspring consisted of 50 % males and intersexes. These males and intersexes were homozygous for a recessive mutant sex determining gene. Inbreeding significantly increased the mean gonad weight as well as the variation in gonad weights. Full sib and heterozygous gynogenetic offspring were normal in gonad development, but gonads from homozygous gynogenetic carp were often retarded in vitellogenesis. The ovulation response was significantly reduced with increasing levels of inbreeding while the numbers of precocious ovulations and non-responders in the 2PB and EM group increased. Homozygous fish were essentially free of recessive lethal genes. Yields of normal fry were reduced in crosses involving eggs from females of the FS and 2PB group when compared to crosses with eggs or milt from EM animals. FS eggs fertilized with EM milt gave significantly better yields of normal fry than any other group. A homozygous inbred strain and two F1 hybrids produced from homozygous gynogenetic offspring were comparable in development but the F1 hybrids showed a clearly reduced variation in both body weight and gonad development. In contrast, the phenotypic variation in the homozygous inbred strain was considerably enlarged for all traits studied.

INTRODUCTION

Gynogenesis is a useful tool for the rapid production of inbred strains of fish. Selected gynogenetic inbred strains and their crosses can be used for stock improvement (Wilkins, 1981; Gjedre, 1988), for standardisation of bio-assays (Falconer, 1981; Richter et al., 1987), and for studies on the regulation of complex biological traits, e.g. the immune response (Kaastrup et al., 1989) or sex determination and differentiation (Komen and Richter, 1990).

However, the genetic control of reproductive and immunological traits in fish and their relationships with growth are still poorly understood, and proper selection criteria for inbred lines are difficult to formulate (Campton and Gall, 1988; Gjedre, 1988).

Furthermore the viability and fertility of homozygous inbred strains produced by gynogenesis has been questioned (Kinghorn, 1983).

Analysis of the phenotypic values of a trait in gynogenetic offsprings with known degrees of inbreeding (F = degree of homozygosity) can provide information on the number of genes involved, the type of interaction between alleles and the presence of deleterious mutations. The mean value of a trait will be depressed in an inbred group as the result of phenotypic expression of unmasked recessive alleles and the reduced frequency of heterozygous loci expressing dominance and overdominance. Concomitantly the variance for this trait will be increased as the result of an increase in homozygous genotypes representing more extreme phenotypic values (Falconer, 1981; Kincaid, 1983).

If gynogenesis is achieved by suppression of the second meiotic division (heterozygous gynogenesis) then the resulting offspring will be homozygous exept for those genes involved in recombination during meiosis (Nace, 1970). The effects of inbreeding for a particular trait in such gynogenetic offspring will therefore depend on the degree of homozygosity for the genes involved. Estimates of F in heterozygous gynogenetic offspring vary from 0.55 in rainbow trout to 0.65 in common carp (Cherfas and Truveller, 1978; Nagy and Csanyi, 1982; Thorgaard et al., 1983; Thompson, 1983).

Gynogenesis by inhibition of the first mitotic division results in duplication of the haploid genome while the first cell division is omitted (endomitosis). The resulting offspring is fully homozygous (homozygous gynogenesis: F = 1) and subsequent gynogenetic reproduction of selected homozygous fish produces a homozygous inbred strain of genetically identical fish ("clone"). Homozygous gynogenetic inbred strains have been produced in zebrafish (*Brachydanio rerio*; Streisinger et al., 1981), medaka (*Oryzias latipes*; Naruse et al., 1985) and recently in common carp (*Cyprinus carpio L.*; Komen et al., 1990a). F1 hybrids produced by crossing these homozygous strains are genotypically identical and essentially free of recessive deleterious genes (Streisinger et al., 1981). They are therefore expected to show a certain degree of heterosis and reduction in variation.

In order to investigate the genetic control of reproductive traits in common carp and to test our assumptions concerning the effects of inbreeding and crossbreeding, we compared the gonad development and fertility in three groups of offspring, produced by full sib breeding (F = 0.25), heterozygous gynogenesis (F = 0.65) and homozygous gynogenesis (F = 1), using the same female. We also present data on the performance

of a homozygous inbred strain and two F1 hybrids produced from homozygous gynogenetic offspring.

MATERIALS AND METHODS

Experimental groups

The female and male used for the production of the experimental groups were full sibs, selected from the progeny of a cross D x W. D and W are animals from a German (D) and Dutch (W) carp strain kept at our laboratory (see also chapter 1). The selected animals were free of any visible abnormalities and could be reproduced successfully. Eggs and milt were obtained and treated as described by Komen et al. (1988; 1990a). The milt stock was diluted 1:3 with ice-cold 0.85 % NaCl and 10 ml was U.V. irradiated (Philips 15 W germicidal tube; 2200 $J/_{m}2_{.min}$ at 253.7 nm) for 60-65 min to inactivate the paternal genome. Heterozygous gynogenetic offspring (second polar body or 2PB group) was produced by giving eggs, fertilized with irradiated milt and incubated at 24 °C, a cold shock (0°C during 45 min) 1-2 min after fertilization. Homozygous gynogenetic offspring (endomitotic or EM group) was produced by giving similarly fertilized and incubated eggs a heat shock (40°C, 2 min), 30 min after fertilization. A full-sib control group (FS group) was produced by fertilizing eggs with untreated milt from the stock solution.

Genetic markers

The selected female was homozygous for a recessive gene determining scattered scalation (mirror carp: s/s), and heterozygous for the two recessive alleles of a duplicated gene involved in melanophore development (b1,+/+,b2). Only homozygous b1,b2/b1,b2 animals have a yellow ("blond") fenotype due to reduced melanophore development (Komen et al., 1990a). The male was heterozygous scaled (wild type: +/s) and normally pigmented. Gynogenetic offspring should contain no scaled fry (no paternal inheritance) and 3-6 % (2PB group) or 25 % (EM group) blond fry. Full sib progeny should consist of normally pigmented fry of which 50 % is scaled.

Husbandry

Fry were raised in 140 l aquaria with recirculating, filtered and U.V.-sterilized water (25 °C). They were fed freshly hatched *Artemia salina* nauplii during the first 3 weeks after hatching, followed by vitamin-C enriched trout pellets (Trouvit, Trouw, Holland) at a daily ration of 30 $g/kg^{0.8}$ body weight. Mortality was only assessed at 8, 15 and 22 weeks after hatching to minimise any damage from handling. Six months after hatching, 200 randomly sampled fish (mean body weight 200 g) from each experimental group were transferred to each of three 800 l rectangular tanks with recirculating water (23 °C). All groups were daily fed trout pellets at 1 % of body weight. The flow rate through each tank was 20 l/min, maintaining the O_2 content above 5 ppm.

Assessment of gonadal development

Gonadal development, expressed as proportional gonad weight (gonado-somatic index : G.S.I.), maturation-stage (% post-vitellogenic eggs) and post-vitellogenic egg weight, was assessed by random sampling 30 fish from each experimental group at 13 months (sample 1) and 19 months (sample 2) after hatching. Fish were killed by electrocution, weighed to the nearest 0.1 g and dissected. Gonads were weighed to the nearest 0.1 g and the G.S.I. calculated as (gonad weight/total fish weight) x 100%. Gonadal sex and sex ratios were determined by macroscopic examination. Gonads were scored as female, male or intersex (gonads containing both testicular and ovarian tissue) and the colour of the eggs was recorded. Two samples of 0.5 - 1.0 g tissue were taken from the middle of the ovary. One sample was fixed in Ca-formol, mounted in paraffin and sectioned at 10 μm. Sections were stained with haemaluin/eosin and classified by counting the numbers of previtellogenic, vitellogenic, post-vitellogenic and atretic oocytes present (Horvath, 1975). The maturation stage was expressed as % post-vitellogenic oocytes per section. The second sample was weighed to the nearest 0.001 g, fixed in Ca-formol and processed by separating and counting post-vitellogenic (yolky) eggs. Egg size was expressed as N (number of eggs) / g (ovary tissue), and calculated as: number of eggs in sample (N) / W sample (g). Samples from male and intersex gonads were taken from the middle of the organ and, in the case of intersex gonads, at the demarcation between ovarian and testicular tissue and processed for histological examination.

Assessment of fertility

Fertility, expressed as ovulation-response after hormonal induction and as fertilization rates and yields of normal fry from ovulated eggs, was determined in randomly sampled females from the FS and the 2PB group at 21 months after hatching. Fish from the EM group could not be sexed with certainty (see results), and were randomly sampled irrespective of their suspected sex. The following procedure was repeated at weekly intervals until 36 fish of each experimental group had been tested.

One group of 6 FS fish and one group of 6 2PB fish were each injected with a priming dose of 0.8 mg carp pituitary suspension (cPS: Hydroquest Int., Rosemont, N.Y) per kg body weight, and a booster dose of either 0.8 mg (control, 2 fish), 1.6 mg (2 fish) or 3.2 mg (2 fish) cps / kg body weight, to induce ovulation. Six EM fish were all injected with 0.8 mg and 3.2 mg cps / kg body weight, since we expected only very few females, who would respond, in this group. The time interval between injections was 30 h. All fish were stripped 11 h after receiving the booster injection (at 23 $^{\circ}$C; Horvath 1978), killed by electrocution and dissected. Stripped eggs, ovulated eggs that could not be stripped, and the ovary itself were all weighed separately to the nearest 0.1 g. The ovulation response was calculated as: (W total ovulated eggs / W (ovary + total ovulated eggs))*100 %. Gonads from non-responding females were sampled for histological examination as already described.

One randomly sampled male from the FS group was injected with a single dose of 1 mg cps / kg body weight and stripped 16 h later. The quality of the stripped eggs from each female, injected with 3.2 mg cps, was determined by fertilizing 100- 200 eggs with sperm from this FS male. Fertilization rate (%) was determined by counting white eggs and developing embryos after 24 h of incubation (at 24°C). The yields of normal and of deformed fry were determined after hatching (96 h) and expressed as: (nr of fry / nr of incubated eggs) * 100 %. The fertility of milt of males from the EM group was determined by fertilizing eggs from FS females.

Homozygous inbred strain and F1 hybrids

A homozygous inbred strain was produced by gynogenetic reproduction of a female, named E_4 from the EM group. Eggs were fertilized with irradiated milt from a FS male and cold shocked (0°C, 45 min) 1-2 min after fertilization. A control group was produced by fertilizing eggs with untreated milt from the FS male. Two F1 hybrids were produced

by fertilizing eggs from the female E_4 with milt of 2 EM males (named E_5 and E_6). The clonal confirmation of the inbred strain and F1 hybrids by skin transplantation has been described elsewhere (Komen et al., 1990b). All groups were raised under standard conditions (see husbandry). At 6 months after hatching 40 fish were randomly sampled from each group, and their length, weight, gonad weight and sex recorded as described.

Parameters and statistical analysis

A Chi-square test for goodness of fit was used to compare observed frequencies of blond fry, males + intersex gonads, and gonads containing yellow eggs with the expected frequencies. The effects of inbreeding on gonad development in sample 1 and sample 2 was assessed by comparing the mean weight, gonad weight, G.S.I., % post-vitellogenic eggs, and egg weight of each experimental group. The homozygous inbred strain, F1 hybrids and control group were likewise compared for differences in mean length, weight, gonad weight and GSI. Log transformed data were tested for homogeneity of variance but did not meet the requirements for analysis of variance (Sokal and Rohlf, 1969). Therefore differences between experimental groups were tested for significance ($P<0.05$) using a Kruskall-Wallis test for k independent samples combined with Wilcoxon's two sample test (SAS). Differences in variation between groups were compared by calculating the coefficient of variation (SD/mean) for each parameter.

Ovulation responses, fertilization rates and yields of normal and deformed fry were transformed using an arc-sin transformation (Sokal and Rohlf, 1969) and analysed with Duncan's multiple range test ($P<0.05$).

RESULTS

The observed frequencies of blond fry in the 2PB group (3 %) and EM group (23.3 %), as well as the observed frequencies of scaled fry in the FS group (51.4 %), were not significantly different from the expected frequencies. There were no scaled fry in the 2PB and EM groups, indicating the absence of paternally derived genes. Mortality between 8 and 15 weeks after hatching was high in the EM group (32.7 %) and the 2PB group (37.0 %), but considerably lower in the FS group (2.3 %). Mortality decreased to 4.5 % in both gynogenetic groups and to less than 1 % in the FS group at 22 weeks after hatching and was negligible in all groups (<1%) in the period thereafter.

Table 1 Frequencies of female, intersex and male gonads, and frequencies of gonads containing yellow eggs, in random sampled fish from the FS, 2PB and EM group. Fish were sampled at age 13 months (sample 1) and at 19 months (sample 2).

group	sexe	sample 1	sample 2	sum[1)]		yellow eggs[2)]		green eggs	
		N	N	N	%	N	%	N	%
FS	♀	20	17	37	61.7	9	24.3	28	75.7
	♀/♂	0	0	0		0		0	
	♂	10	12	22	38.3				
2PB	♀	28	28	56	93.3	0	0	56	100.0
	♀/♂	1	1	2		0		2	
	♂	1	1	2	6.7				
EM	♀	19	13	32	53.3	20	53.2	12	46.8
	♀/♂	8	7	15		5		10	
	♂	3	10	13	46.7				

1 Percentages are calculated from pooled values of male + intersex gonads. The frequencies of males and intersexes in the FS and EM groups were not sign. different from a 50 % ratio according to the Chi-square test ($P<0.05$).
2 Values are from pooled samples. Percentages are calculated from pooled values of female + intersex gonads. The frequencies of gonads with yellow eggs were not significantly different from a 25% ratio (FS group) or 50 % ratio (EM group) according to the Chi-square test ($P<0.05$).

Sex ratios (table 1)

Both samples from the 2PB group contained 28 females, 1 male and 1 fish with intersex gonads. In contrast, both samples from the EM group contained significant numbers of males (3 resp. 10) and fishes with intersex gonads (8 resp. 7; see table 1). Assuming the intersex gonad as a phenotype produced by the same mutation(s) as testis (see discussion), the numbers of intersex and male gonads from both samples were pooled. The obtained frequency (46.7 %) was not significantly different from an expected 50 % ratio (see table 1). The pooled frequency of males in the FS group was 38.3 %. This deviation from the 50 % ratio was due to the removal of males during the rearing period. Fishes with intersex gonads were not found in this group.

Gonadal development females (table 2)

There were no significant differences between females of the FS, 2PB and EM group

concerning mean body weight at 13 months. The relative increase in body weight between samples was less for fish from the EM group than for fish from the 2PB or the FS group, resulting in a significant lower body weight at 19 months for this group.
There were significant differences in gonad development between experimental groups. Fish from the FS group had a significantly lower gonad weight and GSI than gynogenetic fish at 13 months after hatching. At 19 months the EM group had a significant lower mean gonad weight than the 2PB group, while differences between FS and 2PB group and between FS and EM group were insignificant. Differences for mean GSI between groups at 19 months were similar but insignificant. The FS group realised the largest increment in GSI between samples (97.6 %), followed by the 2PB group (58.2 %) and the EM group (27.1 %).
The gonads of females from the 2PB and FS group were similar in maturation upon histological examination. At 13 months after hatching they contained numerous pre-vitellogenic oocytes (stage I-III) and only a few vitellogenic oocytes (yolk formation stage IV-VI; Horvath, 1985). Post vitellogenic (yolky) oocytes comprise 18.6 - 22.1 % of the total number of oocytes present (mean values, see table 2). At this age the increase in gonad weight is in part due to an increase of post vitellogenic eggs (fig 1a and 1b). At 19 months ovaries of FS and 2PB fish contain between 20 and 40 % post vitellogenic oocytes, but 2PB fish show a larger variation in both gonad weight and numbers of post vitellogenic eggs compared to FS fish (compare table 2 and fig 1a and 1b). Gonadal development of EM females could be classified as normal or retarded. Normally developed ovaries were similar to those in FS and 2PB fish. Retarded ovaries contained large numbers of vitellogenic oocytes and only a
few post vitellogenic oocytes (<5%; see fig 1). At 19 months the difference between normal and retarded gonads was even more pronounced. Retarded gonads still contained less than 15 % post vitellogenic oocytes, despite their often large size (fig 1c).
Eggs from EM fish were smaller than eggs from gonads of 2PB and FS fish (both samples), but these differences were not significant. There was a considerable increase in egg weight between samples for all groups. Some females from the FS group possessed gonads with yellow eggs instead of green. Such gonads did not occur in the 2PB group but in the EM group 20 females and 5 intersex fish with yellow eggs were found. The pooled frequencies for yellow eggs in the EM group (53.2 %) and FS group (24.3 %: table 1) were not significantly different from a 50 % or 25 % ratio.

Table 2 Mean values and coëfficiënt of variation of various morphological parameters for females in random samples from the FS, 2PB and EM group, and the relative increase for the mean of these parameters between samples ($\delta\%$) (see table 1).

parameter	group	sample 1 (13 months)			sample 2 (19 months)			δ%
		mean	SD	C.V.	mean	SD	C.V.	
body weight (g)	FS	786.9	225.3	28.6	1353.0^{ab}	285.7	21.1	71.9
	2PB	810.8	337.8	41.7	1598.0^{b}	525.8	32.9	97.1
	EM	754.5	226.8	30.1	1164.1^{a}	448.0	38.5	54.3
ovary weight (g)	FS	72.8^{a}	43.3	59.5	247.8^{ab}	63.6	25.7	240.6
	2PB	107.6^{ab}	66.0	61.3	291.0^{b}	124.5	35.1	170.5
	EM	101.6^{b}	48.5	47.7	209.5^{a}	102.2	59.4	106.7
GSI	FS	9.3^{a}	4.0	43.0	18.3	3.0	16.4	97.6
	2PB	11.9^{b}	4.9	41.2	18.9	4.3	22.8	58.2
	EM	13.4^{b}	5.2	38.8	17.0	6.9	40.6	27.1
post vit. eggs (%)	FS	18.6^{a}	7.4	39.8	29.0^{a}	4.7	16.2	56.2
	2PB	22.1^{a}	11.2	50.7	31.3^{a}	8.0	25.6	41.6
	EM	9.9^{b}	7.0	70.7	18.0^{b}	12.0	66.7	80.8
egg size (N / g)	FS	2094^{a}	550	26.3	1378	157	11.4	-34.2
	2PB	2273^{ab}	897	39.5	1368	201	14.7	-42.0
	EM	2642^{b}	778	29.5	1586	480	30.3	-40.0

Mean values for groups within a sample with common superscripts are not sign. different according to Wilcoxons two sample test ($p < 0.05$). Coefficient of variation was calculated as (SD / mean) * 100 %.
The relative increase between samples was calculated as:
(mean sample 1 - mean sample 2) / (mean sample 2)

Table 3 Mean values and coëfficiënt of variation for various morphological parameters of males in random samples from the FS and EM group, and the increase for these parameters between samples (δ%).(see also table 1)

parameter	group	sample 1			sample 2			δ%
		mean	SD	C.V.	mean	SD	C.V.	
body weight (g)	FS	676.9*	118.5	17.5	1296.0*	174.9	13.5	91.5
	EM	378.6	201.4	53.2	808.4	286.6	35.5	113.5
testis weight (g)	FS	43.9*	17.1	39.0	135.4*	43.0	31.8	208.8
	EM	15.3	9.4	61.4	63.5	43.0	67.7	314.4
GSI	FS	6.5	2.0	30.8	10.5*	3.1	29.8	63.2
	EM	4.1	2.2	53.6	7.6	5.3	69.6	83.3

* = mean values for groups within a sample are significantly different according to Wilcoxons two sample test ($p < 0.05$). For further explanation see table 2.

The coëfficient of variation (C.V.) decreased between samples for all parameters in both the FS and 2PB group but increased for fish from the EM group. At 19 months, C.V. was largest for the EM group, followed by the 2PB group, and smallest for the FS group.

Gonad development males + intersexes (table 3)

EM males (i.e. no intersex gonads) were significantly smaller than FS males and possessed significantly smaller testis. (63.5 vs 135.4 g; sample 2). The relative rate of increase for these characters was larger for EM males than for FS males. There was also more variation in size and testis weight between males of the EM group when compared to the FS males. Testis of FS males all contained large tubuli seminiferi, filled with spermatozoa, whereas testis from EM males were less developed, containing only mature cysts which are occasionally fused.

Table 4 Ovulation responses of common carp females from the FS, 2PB and EM group after injection with 0.8, 1.6 or 3.2 mg/Kg of carp pituitary suspension to induce ovulation. Fish were sampled at age 21-23 months.

group	total	0.8 mg cPS/Kg					1.6 mg cPS/Kg					3.2 mg cPS/Kg				
		m	-	ovulation			m	-	ovulation			m	-	ovulation		
	N	N	N	N	%	(sd)	N	N	N	%	(sd)	N	N	N	%	(sd)
FS	36	0	11	1	13.4	(-)	0	0	12	60.5^{b}	(19.3)	0	1	11	63.3^{b}	(13.8)
2PB	34	3	8	0	-		2	2	8	40.4^{a}	(24.4)	2	2	7	55.5^{ab}	(25.1)
EM	20											3	11	6	48.9a	(21.2)

m = missing; - = not responding; % refer to mean ovulation response (± sd)
Values with identical superscript are not significantly different according to Duncan's multiple range test ($P<0.05$).

Fish with intersex gonads were comparable to females with respect to mean body weight (1145.2 in sample 2; compare with table 2), but gonad weight and G.S.I. were highly variable. All intersex gonads were characterised by clearly demarcated areas of testicular and ovarian tissue. The ratio of male to female tissue varied from 10:90 to 90:10 (%) in both samples. Testicular development was comparable to testis development in EM males while ovarian tissue was usually inhibited, containing mainly vitellogenic and atretic oocytes.

Ovulation response (table 4)

All selected FS fish were females, but 2 intersexes were found in the 2PB group. Fish from the EM group consisted of 20 females and 16 males and intersexes.

Only 1 FS fish ovulated after recieving a control dose of 0.8 mg cps. All 12 FS fish and 8 2PB fish were stripped after injection with 1.6 mg cps. Two 2PB fish ovulated before recieving a booster dose and 2 fish did not ovulate. The ovulation response of FS fish was significantly better (60.5 ± 19.3) than of 2PB fish (40.4 ± 14.4) at this dose. Fish injected with 3.2 mg cps reacted in a comparable way. Eleven FS and 7 2PB fish were

Table 5 Survival of embryos 24 h after hatching, and yields of normal an deformed fry, 96 h after hatching, from crosses between FS, 2PB and EM females, and FS and EM males. Fish were induced to ovulation by injection with carp pituitary suspension (3.2 mg cps/Kg: see also table 4).

CROSS	N	survival t24 embryos (%)		survival t96 normal fry (%)		survival t96 deformed (%)	
♀ x ♂		mean	(SD)	mean	(SD)	mean	(SD)
FS x FS	11	92.3	(5.5)	79.8	(10.4)	10.2	(8.4)
2PB x FS	7	83.7	(20.7)	73.5	(21.7)	3.8	(2.5)
EM x FS	6	92.3	(3.6)	80.9	(3.6)	3.7	(2.6)
FS x EM	9	93.6	(4.7)	88.8*	(4.8)	2.1	(1.7)

One EM female did not produce sufficient eggs and was excluded from this experiment.
Mean survival was calculated from total incubated eggs.
* = yields of normal fry were significantly different according to Duncan's multiple range test.

strippable while 1 FS and 2 2PB fish were not. Two 2PB fish ovulated before the booster injection. Of the 20 EM fish only 6 responded. One fish died during the experiment, 2 fish ovulated before the booster and 10 fish did not react. One fish ovulated but could not be stripped due to malformations of the gonadal duct.

The ovulation response was better in FS fish than in 2PB fish (63.3 vs 55.5 %, table 4) and significantly reduced in EM fish (48.9 ± 21.2 %). Ovaries of non responding FS and 2PB females were normally developed and showed signs of maturation, i.e. germinal vesicle migration and germinal vesicle dissolution. In contrast ovaries from EM females who did not ovulate after the cps treatment were all retarded in development and similar to the gonads already described.

Fertility (table 5)

All egg samples from the EM and FS fish had good fertilization rates (92.3-93.6). There

was a large variation in fertilization rates of eggs from 2PB fish. Yields of normal fry were reduced and highly variable in FS x FS and 2PB x FS batches when compared to crosses with EM eggs or milt.
FS eggs fertilized with EM milt had significantly better yields of normal fry than any other group. Rates of deformed fry were higher in FS x FS batches than in the other batches but this difference was not significant.

Table 6 Sex and mean length, weight, gonad weight and GSI of fishes from a homozygous inbred strain and two F1 hybrids of common carp, 24 weeks after hatching (at 25 °C).

GROUP	All fish in sample									All females in sample					
	sex (n)			Length (cm)			Weigth (g)			Gonad W. (g)			GSI		
	♀	♀/♂	♂	mean	± sd	CV	mean	± sd	CV	mean	± sd	CV	mean	± sd	CV
E4 gyn	38	1	0	14.8	2.7	18	151.5	64.3	42	0.58	0.47	81	0.33	0.21	64
E4 x E5	37	2	0	17.5	0.8	5	194.2	29.8	15	0.99	0.29	29	0.52	0.16	31
E4 x E6	39	0	1	16.6	1.4	9	167.2	37.7	23	1.00	0.33	33	0.61	0.22	36
E4 x FS	17	0	23	17.9	1.3	7	219.8	45.9	21	0.61	0.27	44	0.29	0.11	38

One EM female E4 and 2 EM males E5 and E6 were used to produce the various strains. The EM female was also crossed with a FS male to produce a normal control offspring.

Homozygous inbred strain and F1 hybrids (table 6)

The homozygous inbred strain and F1 hybrids consisted mainly of females and one or two intersexes. The E_4 x FS strain contained 17 females and 23 males. This group had the largest mean length and weight after 6 months, but the differences between groups were not significant. The F1 hybrids E_4 x E_5 and E_4 x E_6 both had higher gonad weights and GSI's than the other groups (not significant). Fishes of the E_4 x E_5 strain and E_4 x E_6 strain were also less variable in gonad weight and GSI than fishes of the E_4 x FS group. The largest variation for all characters was found in fishes of the homozygous inbred strain.

DISCUSSION

The effects of inbreeding on various reproductive traits could be classified as single recessive gene effects and differences in the mean and variation of a trait between groups.

The most striking effect caused by a single gene was the occurrence of 46.7 % males and intersexes in the EM group, and the virtual absence of such fish in the FS and 2PB group. In common carp males are thought to be XY and females XX since conventional breedings consistently produce 50 % males while gynogenesis produces all female offspring (Nagy et al., 1978; Komen, unpublished results). Nevertheless, intersex gonads are occasionally noted in conventional offspring (Gupta and Meske, 1976; Hilge and Conrad, 1975) and in large numbers in some heterozygous gynogenetic offspring (Gomelskii et al., 1978). Such intersexes, as well as the males and intersexes found in this study, probably have a genetic origin (Komen et al., in prep). It is assumed that the selected female progenitor used in this study was heterozygous for a recessive mutation in a minor sex determining gene, termed mas-1. In homozygous condition this mutation induces a testis or (incomplete penetrance) an intersex gonad in XX offspring. Normal female sex differentiation is restored in heterozygous offspring from (mas-1/mas-1) EM males crossed with (+/+) EM females (see also table 6), but a conventional cross between an EM male and an heterozygous (mas-1/+) female produces again 50 % intersexes and males in the offspring (Komen and Richter, 1990). The near absence of males and intersexes in the 2PB group argues for a high degree of heterozygosity for mas-1 in this group.

A similar high degree of heterozygosity was also found for another new mutant discovered in this study, termed yellow eggs (ye). Yellow eggs are a common feature of fancy (ornamental) carp, but the mode of inheritance is unknown (S. Rothbard, pers. comm.). In our experiments yellow eggs were found both in the FS and EM group, but not the 2PB group. The obtained frequencies (24.3 and 53.2 %, see table 1) are consistent with a monogenic recessive inheritance for this trait, and indicate 100 % heterozygosity for ye in the 2PB group. The high recombination rates found for mas-1 and ye are typical for common carp and several other fish species, and are probably caused by high levels of interference in a number of chromosomes, resulting in single obligatory chiasmata during meiosis (Thorgaard et al., 1983; Thompson and Scott, 1984;

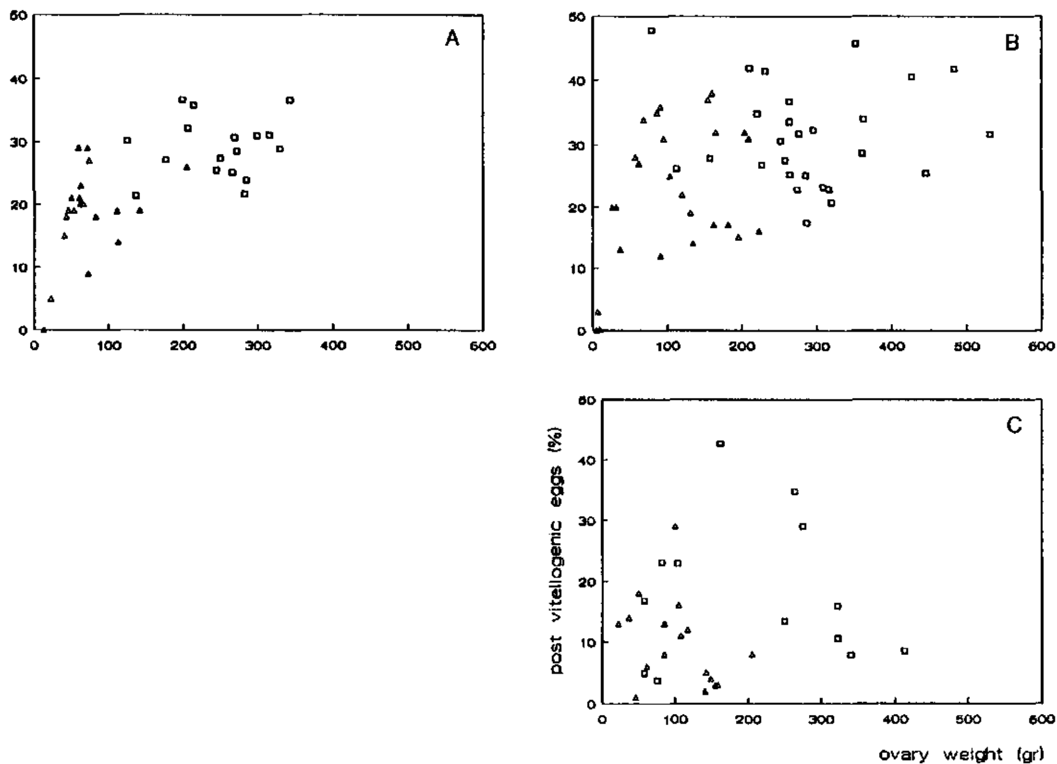
Streisinger et al., 1986). In consequence 2PB offspring remain heterozygous, and thus identical, for those genes which are located distally from these chiasmata. A similar conclusion was drawn when the rejection times of skin allografts grafts exchanged within a FS, 2PB and EM group of carps were compared (Komen et al., 1990b). Graft rejection was considerably delayed in the 2PB group in comparison to graft rejection among FS or EM fish, indicating a high degree of genetic similarity for 2PB fish.

Gonad development

These observations might help to explain why inbreeding depression for gonad devlopment was only noticed in the EM group while fish from the 2PB group were comparable with fish from the FS group (see table 2). Only at 13 months the EM and the 2PB group showed a significantly higher GSI and gonad weight than the FS group.
Gonad weight during sexual maturation is the result of fecundity (the total number of oocytes in the gonad) and vitellogenesis (the relative number of post-vitellogenic oocytes). In the platyfish (*Xiphophorus maculatus*) the onset of vitellogenesis and subsequent maturation of the gonad is determined by a sex chromosome linked locus which controls the development of the pituitary-gonadal axis (Kallman and Borowski, 1976; Schreibman and Kallman, 1977). The alleles of this locus (at least 5) act in a more or less dominant

fashion. It has been suggested that sexual mturation in common carp is regulated in a similar way (Hulata et al., 1985). Early maturing Chinese "Big Belly" carp have high gonad weights while various late maturing european strains have lower gonad weights. Crossbreeding between these strains revealed incomplete dominance for later maturation and lower gonad weights (Hulata et al., 1974, 1985). In this study the higher mean gonad weight and GSI of 2PB fishes might be explained by a reduction in heterozygosity for the loci controlling gonadal growth, while the increased variation is due to the increased frequency of homozygous genotypes producing more extreme gonad weights and GSI's (Falconer, 1981). The gonad weights at 13 months are even higher but become increasingly depressed by a reduced vitellogenesis in nearly half the homozygous EM fish. It is possible that this reduced vitellogenesis is in fact the manifestation of an unmasked recessive deleterious gene. Alternatively these fish might represent late maturing genotypes. In that case the rate of vitellogenesis and sexual maturation in common carp is controlled in a similar way as in platyfish by a single locus with dominant alleles. The

Figure 1 The relationship between the relative presence of post vitellogenic eggs (%) and gonad weight in random sampled females from the FS, 2PB and EM group. Fish were sampled at 13 months ( ) and 19 months ( ) after hatching.
A) FS group; B) 2PB group; C) EM group.



recombination frequency for this locus might be high since FS and 2PB fish were fully comparable in development. Taken together, these results clearly show that inbreeding increases the mean proportional gonad weight by or in combination with an advanced early maturation. Selection for late maturing genotypes with low gonad weights will be feasible only in EM offspring.

Fertility

More classical and straightforward effects of inbreeding were found in the fertility test. The ovulation response was significantly reduced with increasing levels of inbreeding while the numbers of precocious ovulations and non-responders in the 2PB and EM groups clearly indicated an increased sensitivity to stress. The selection involved with inbreeding was illustrated by the results of the various crosses between FS, 2PB and EM fish. Again a large number of deleterious mutations in genes involved in embryo development remain heterozygous in FS and 2PB offspring and thus escape selection by mortality. The persistence of such genes in heterozygous gynogenetic offspring was also found by Nagy (1987) who detected high levels of embryo malformations after crossing hormonally sex inversed males from a fourth (!) gynogenetic generation with females from a third gynogenetic generation. These results do not justify a use of 2PB gynogenetic females for the production of homozygous gynogenetic fry by endomitosis, as was suggested by Chourrout (1987). We suggest on the contrary the use of crossbred females from two distinct strains for this kind of gynogenesis. Not only are the eggs of such females more viable and more uniform in quality, leading to more consistent yields of homozygous gynogenetic fry (Komen et al., 1990), but the homozygous offspring will also present a much larger pool of genetic variation to select from.

Inbred strain and F1 hybrids

Homozygous gynogenetic fish are essentially free of recessive lethals as was demonstrated for zebrafish (*Brachydanio rerio*; Streisinger et al., 1981) and in this study for common carp by a significantly higher yield of normal fry and very few malformations in crosses involving homozygous animals. Embryos were less viable in crosses involving EM eggs compared to crosses where EM milt was used but in general these results showed that eggs and milt from homozygous gynogenetic fish were fully fertile. Homozygous gynogenesis is thus an effective way of producing inbred strains.

The F1 hybrids E_4 x E_5 and E_4 x E_6 were comparable in development to the E_4 x FS strain but showed an important reduction in variation for gonad development. In contrast this purely phenotypic variation was enlarged for all characters in the homozygous inbred strain. In this respect inbred strains of common carp behave like any other inbred strains (Falconer, 1981) although the observed variation is considerable when compared to other vertebrate species (Allendorf et al.,1988). This augmented variation is often attributed to reduced homeostasis i.e. the inability of an organism to develop along precisely predetermined pathways (Lerner, 1954). Developmental instability is expressed as an increase in asymmetry for various morphological traits. Studies in rainbow trout have shown that individuals with developmental instability are often homozygous for enzyme variants involved in major biochemical pathways (Leary et al., 1983). In this study deformed fish were also found in the homozygous inbred strains while they did not occur in the F1 hybrids. However, this may be caused by food availability and crowding, since deformed fish only occurred in the lower weight classes. These fish were probably less successful in competing for food and more susceptible to the negative effects of crowding. It is therefore concluded that F1 hybrid strains will be more suitable for use in bio-assays than homozygous inbred strains, since F1 hybrids are expected to show less variable and more standardized responses.

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Chapter 7

EFFECTS OF ORAL ADMINISTRATION OF 17α-METHYLTESTOSTERONE AND 17ß ESTRADIOL ON GONADAL DEVELOPMENT IN COMMON CARP, CYPRINUS CARPIO,L

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Effects of Oral Administration of 17α-Methyltestosterone and 17β-Estradiol on Gonadal Development in Common Carp, *Cyprinus carpio* L.

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ABSTRACT

Komen, J., Lodder, P.A.J., Huskens, F., Richter, C.J.J. and Huisman, E.A., 1989. Effects of oral administration of 17α-methyltestosterone and 17β-estradiol on gonadal development in common carp, *Cyprinus carpio* L. *Aquaculture*, 78: 349-363.

The effects of oral administration of 17α-methyltestosterone (17α-MT) and 17β-estradiol (17β-E2) on the gonadal development of common carp were investigated during periods of 5 weeks between 3 and 15 weeks after hatching. Administration of 50 ppm 17α-MT in the food between 6 and 11 weeks after hatching resulted in 92.7% males. Earlier or later treatments with 17α-MT at concentrations of 50 and 100 ppm of hormone in the food resulted in high percentages of sterile fish. Administration if 17β-E2 failed to induce female gonads in any of the periods tested and irrespective of the concentrations of hormone used. Gonad weight and gonadosomatic index of both males and females decreased with increasing doses of 17β-E2, indicating that the hormone had been absorbed by the fish. For hormonal sex control in other fish species it is recommended to apply hormone dosages which are related to body weight and not to body weight gain.

INTRODUCTION

Control of sex differentiation by administration of sex steroids plays an important role in the culture of various fish species. It can be used to create monosex populations in ponds if natural spawning is not desired (tilapias: Shelton et al., 1981; Rothbard et al., 1983) or if growing of one sex has certain advantages over the other (salmonids: Johnstone et al., 1978; Donaldson and Hunter, 1982). In combination with gynogenesis, it can be used to establish inbred lines with fish of either sex (Nagy and Csanyi, 1984). The development of gynogenetically inbred lines of fish could also serve to standardize bioassays in immunological and endocrinological research (Van Muiswinkel et al., 1986; Richter et al., 1987; Komen et al., 1988).

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There are few reports concerning hormonal sex induction in common carp, *Cyprinus carpio*. Nagy et al. (1981) successfully induced male gonads in gynogenetic females, using 100 ppm 17α-methyltestosterone incorporated in the food, administered during any period of 36 days between 8 and 80 days after hatching. Sathyanarayana Rao and Satyanarayana Rao (1983) obtained high percentages of male and sterile gonads in *C. carpio* after oral administration of 220 ppm 17α-MT during 131 days after hatching. Identical treatment with 17β-estradiol was less successful, since sterile gonads and gonads of both sexes were recovered. These results confirm the importance of both dosage and timing in treatments with sex steroids (Yamamoto, 1969).

To obtain gynogenetic homozygous lines with phenotypic female and male fish, we investigated the effects of oral administration of 17α-methyltestosterone (17α-MT) on the gonadal development of common carp. In addition, similar experiments were carried out with 17β-oestradiol (17β-E2) to obtain XY females. These fish could give information on the expression of male sex chromosomes in sex-induced females (Hunter and Donaldson, 1983).

In the present experiments, three different periods (3–8, 6–11 and 10–15 weeks) after hatching were tested, each with different concentrations of 17α-MT and 17β-E2.

MATERIALS AND METHODS

Experimental fish

Fry for both experiments were obtained by the method of artificial propagation as described by Woynarovich (1962). For each experiment eggs and milt from one female and one male were used.

The fry were fed freshly hatched nauplii of *Artemia salina* for a period of 2 weeks following yolk-sac absorption. During the experimental period, the fish were kept at 25°C (Horvath, 1985) and fed trout pellets (45% protein, Trouvit, The Netherlands) using Scharfflinger conveyer-belt feeders, for 12 h a day. The fish were sampled and weighed every week. The mean biomass and mortality were estimated, and the feeding level adjusted according to the recommendations given by Huisman (1976). At this feeding level, all the food was eaten immediately after administration.

Experimental diets

Experimental diets containing 17α-MT or 17β-E2 (Intervet, Boxmeer, The Netherlands) were prepared by the alcohol evaporation method (Guerrero, 1975). Depending on the pellet size, 1 kg of pellets was carefully mixed with 400 (size 1 and 2), 550 (size 0) or 750 (size 00) ml of 96% ethanol containing 50, 100 and 150 mg 17α-MT. In the same way, pellets containing 25, 75 and

125 mg 17β-E2 per kg were prepared. The wet pellets were dried for 24 h at room temperature. Control diets consisted of pellets, mixed with ethanol only. The diets were stored at 4 °C and administered within 4 days after preparation.

Experimental design

First experiment. Three weeks after hatching, 500 fry were stocked in each of sixteen 65-l aquaria. The aquaria were part of a recirculation system in which the O_2 content was maintained above 6 ppm and the NH_4 and NO_2 content below 1 ppm. Three different periods of treatment were tested, each with different concentrations of 17α-MT in the food:

- period I: 3 to 8 weeks after hatching; 50 and 100 ppm
- period II: 6 to 11 weeks after hatching; 50, 100 and 150 ppm
- period III: 10 to 15 weeks after hatching; 50 and 100 ppm.

The second period carried control groups. These fish were fed control diets.

Second experiment. Three weeks after hatching, 500 fry were stocked in each of twenty 140-l aquaria. The lower density was chosen to fit the lower capacity of the recirculation system. The O_2 content wa maintained above 6 ppm and the NH_4 and NO_2 content below 2 ppm. Again three periods of treatment were tested with different concentrations of 17β-E2 in the food:

- period I: 3 to 8 weeks after hatching; 25 and 75 ppm
- period II: 6 to 11 weeks after hatching; 25, 75 and 125 ppm
- period III: 10 to 15 weeks after hatching; 25 and 75 ppm

Each test period carried control groups.

In both experiments, all combinations were tested in duplicate and randomly assigned to each of the aquaria. The water from aquaria with fish receiving hormone was not recirculated during the periods of treatment. Eight weeks after hatching, the stocking density was reduced to 100 randomly sampled fish per aquarium. Fifteen weeks after hatching, the stocking density was further reduced to 50 fish per aquarium. Twenty-four weeks after hatching, the experiments were terminated and the fish sacrificed for dissection and histological examination.

Parameters used to assess effects of hormonal treatment

Fish were killed with an overdose of ethyleneglycol monophenylether (Merck), and weighed to the nearest 0.1 g. Fish that produced sperm after stripping were scored as such. The fish were dissected and the gonads were weighed to the nearest 0.01 g. The gonadosomatic index (GSI) was calculated as (gonad weight/total weight) $\times$ 100%. Gonadal sex was determined using a low power microscope (40$\times$, Zeiss). Gonads having both testicular and ovar-

ian tissue were scored as intersex, while filiform gonads were scored as sterile. These types of gonads and gonads of which the sex could not be determined were fixed in Ca-formol, sectioned and stained with haemalum/eosin for histological examination.

Effects of hormonal treatments were assessed as: mortality rate; increase in body weight; frequencies of male, female, intersex and sterile gonads; increase in gonad weight and GSI; and frequencies of males that could be stripped. These data were determined per dosage and period.

Statistical analysis

Log-transformed data were tested for homogeneity of variance and normality (BMDP, Dixon 1983), but did not meet the requirements for analysis of variance (Sokal and Rohlf, 1969). Therefore data from duplicate groups were pooled and differences in body weight, gonad weight and GSI between treatments within each tested period were analysed using a Kruskall–Wallis test for k independent samples ($P=0.05$). Testing for differences between periods was not found meaningful due to differences in experimental conditions caused by mortality. A chi-square test for heterogeneity with Yates correction was used to determine whether frequencies in occurrence of males differed from the expected 50% ratio and to compare differences in percentages of males shedding milt within the same treatment periods (BMDP, Dixon 1983).

RESULTS

First experiment

Mortality of untreated fish between 3 and 8 weeks after hatching ranged from 16 to 30%, while severe lordoses and other malformations occurred in fish fed 50 and 100 ppm 17α-MT, leading to mortalities of 28% and 39% respectively. Mortality was less than 1% between 8 and 24 weeks after hatching and malformations as a result of hormonal treatment did not occur.

The mean body weight at the end of the experiment was not significantly different between hormone-treated groups and the control group ($P<0.05$) for treatment period II (see Table 1). Differences in mean body weight within treatment periods were also not observed at the end of the experiment ($P<0.05$). A comparison between the different treatment periods shows that the same concentrations of 17α-MT in the food are not proportional to the total dosage of hormone expressed as mg/kg body weight and are reasonably proportional to the total dosage of hormone expressed as mg/kg body weight gain (Table 1). These observations are a consequence of the feeding regimes used (Huisman, 1976); see also discussion.

The frequencies in occurrence of males were, with the exception of the con-

TABLE 1

Concentrations of 17α-methyltestosterone in the food, dosages of hormone per kg body weight and per kg body weight gain administered to common carp (*C. carpio*) at various periods after hatching

Treatment		Start treatment		End treatment		Total treatment	End experiment
Period	17α-MT (ppm)	Mean weight (g)	Dosage (mg/kg body weight)	Mean weight (g)	Dosage (mg/kg body weight)	Dosage (mg/kg weight gain)	Mean weight (g) (SD)
I	50	0.02	10	2.08	7.5	44.7	93.4 (40.0)
	100	0.02	20	2.33	15	86.4	95.3 (41.1)
II	0	0.92	-	11.86	-	-	102.4 (39.2)
	50	0.90	7.5	11.92	4	48.3	91.5 (34.1)
	100	0.92	15	11.82	8	103.5	96.7 (33.8)
	150	0.99	22.5	12.33	12	160.4	92.1 (37.7)
III	50	11.97	4	30.51	2	57.3	89.3 (35.2)
	100	11.87	8	30.77	4	116.9	88.9 (36.5)

Values are from pooled duplicates and were compared within the same treatment periods. There were no significant differences in body weight according to the Kruskal–Wallis test ($P<0.05$).

TABLE 2

Frequencies of male, female, intersex and sterile gonads in common carp, after various dietary treatments with 17α-MT (see also Table 1)

Period	17α-MT (ppm)	Number of fish	Males (%)	Females (%)	Intersex (%)	Sterile (%)
I	50	98	37.8[a]	0	0	62.2
	100	99	7.1[b]	0	0	92.8
II	0	90	64.4[a]	32.2	0	3.3
	50	96	92.7[c]	2.1	0	5.2
	100	91	80.2[b]	3.3	2.2	14.3
	150	97	76.3[a]	7.2	5.2	11.3
III	50	95	74.7[a]	5.3	1.1	18.9
	100	97	80.4[a]	8.2	0	11.3

Values within a period with identical superscripts were not significantly different according to the chi-square test ($P<0.05$). The frequencies of males in all groups, except the control groups, were significantly different from the expected 50% ratio ($P<0.05$).

TABLE 3

Gonad weight, GSI and percentages of male common carp shedding milt, after various dietary treatments with 17α-MT (see also Tables 1 and 2)

Treatment		All fish in sample		All males in sample		
Period	17α-MT (ppm)	Mean gonad weight (g) (SD)	GSI mean (SD)	Mean gonad weight (g) (SD)	GSI mean (SD)	Shedding milt (%)
I	50	0.96 (1.93)[a]	0.99 (1.71)[a]	2.37 (2.48)	2.43 (1.99)	10.3[a]
	100	0.18 (0.73)[b]	0.19 (0.69)[b]	2.21 (1.87)	2.35 (1.40)	0[a]
II	0	1.63 (1.77)[a]	1.72 (1.69)[a]	2.21 (1.96)	2.42 (1.75)	43.1[b]
	50	2.28 (1.71)[b]	2.55 (1.73)[b]	2.44 (1.68)	2.73 (1.67)	4.0[a]
	100	2.05 (1.84)[ab]	2.06 (1.87)[a]	2.46 (1.80)	2.48 (1.84)	19.7[a]
	150	1.73 (1.83)[a]	1.87 (1.68)[a]	2.16 (1.89)	2.34 (1.65)	2.7[a]
III	50	1.04 (1.26)[a]	1.26 (1.38)[a]	1.32 (1.34)	1.63 (1.42)	9.9[a]
	100	0.92 (1.09)[a]	1.03 (0.96)[a]	1.10 (1.15)	1.24 (0.96)	5.1[a]

Values within a period and column with identical superscripts were not significantly different according to the Kruskal-Wallis test (gonad weight and GSI; $P<0.05$) and the chi-square test (% of males shedding milt; $P<0.05$).

trol group, significantly different from the expected 50% ratio (Table 2). The lowest percentage of male gonads was found in the first treatment period. Apparently, 17α-MT had a sterilizing (see also histological examination) effect on gonad development in the period 3–8 weeks after hatching. The highest percentage of male gonads was found in the fish fed 50 ppm 17α-MT during the second period. With increasing concentrations of 17α-MT in the food (period II) the percentage of female, intersex and sterile gonads increased (Table 2).

There was a statistically significant decrease in gonad weights and GSI of all the fish in the sample within periods I and II (Table 3) with increasing concentrations of 17α-MT in the food. A similar tendency was observed when only the males were considered but these differences were not statistically significant. The percentage of males that shed milt after stripping was significantly reduced by hormonal treatment (see period II in Table 3).

Second experiment

All groups suffered high mortalities between 3 and 8 weeks after hatching, ranging from 51 to 69%. Mortalities between 8 and 24 weeks after hatching were less than 1% for all groups. Malformations as a result of treatment with 17β-E2 were not observed.

TABLE 4

Concentrations of 17β-estradiol in the food, dosages of hormone per kg body weight and per kg body weight gain administered to common carp at various periods after hatching

Treatment		Start treatment		End treatment		Total treatment	End experiment
Period	17β-E2 (ppm)	Mean weight (g)	Dosage (mg/kg body weight)	Mean weight (g)	Dosage (mg/kg body weight)	Dosage (mg/kg weight gain)	Mean weight (g) (SD)
I	0	0.05	-	4.04	-	-	87.3 (52.1)
	25	0.07	5	3.39	3.8	-	82.1 (52.1)
	75	0.05	15	2.03	11.3	-	78.2 (55.7)
II	0	0.43	-	15.72	-	-	80.4 (41.6)
	25	0.44	3.8	13.16	2	27.5	75.8 (38.5)
	75	0.40	11.3	13.94	6	82.5	84.8 (46.6)
	125	0.45	18.8	13.49	10	137.5	80.6 (44.7)
III	0	11.20	-	40.73	-	-	83.4 (48.7)
	25	7.60	2	31.55	1	35.3	75.6 (37.7)
	75	7.90	6	25.41	3	105.8	75.4 (42.2)

Values are from pooled duplicates and were compared within the same treatment periods. There were no significant differences in body weight according to the Kruskal-Wallis test ($P<0.05$).

TABLE 5

Frequencies of male, female, intersex and sterile gonads of common carp, after various dietary treatments with 17β-E2 (see also Table 4)

Period	17β-E2 (ppm)	Number of fish	Males (%)	Females (%)	Intersex (%)	Sterile (%)
I	0	73	53.4	45.2	1.4	0
	25	99	50.5	44.4	4.1	1.0
	75	95	62.1	33.7	2.1	2.1
II	0	96	58.3	40.6	1.1	0
	25	99	42.4	53.5	4.1	0
	75	99	46.5	49.5	3.0	1.0
	125	92	53.3	44.6	2.1	0
III	0	99	50.5	46.5	3.0	0
	25	98	48.0	50.0	1.0	1.0
	75	100	45.0	50.0	5.0	0

The frequencies of males and females were not significantly different between hormone-treated groups and their controls, and were not significantly different from the expected 50% ratio, according to the chi-square test ($P<0.05$).

TABLE 6

Gonad weight, GSI and percentages of male common carp shedding milt, after various dietary treatments with 17β-E2 (see also Tables 4 and 5)

Treatment		All females in sample		All males in sample		
Period	17β-E2 (ppm)	Mean gonad weight (g) (SD)	GSI mean (SD)	Mean gonad weight (g) (SD)	GSI mean (SD)	Shedding milt (%)
I	0	0.96 (0.82)^{a}	1.10 (0.57)^{a}	3.66 (2.16)^{a}	4.28 (1.71)^{a}	51.3^{a}
	25	0.79 (0.51)^{a}	1.07 (0.33)^{a}	3.49 (3.38)^{ab}	3.65 (1.88)^{a}	28.0^{b}
	75	0.59 (0.39)^{b}	0.79 (0.26)^{b}	2.83 (2.41)^{b}	3.65 (2.13)^{a}	15.3^{b}
II	0	0.76 (0.50)^{a}	0.94 (0.38)^{a}	3.31 (3.23)^{a}	3.86 (2.04)^{a}	50.0^{a}
	25	0.62 (0.37)^{a}	0.92 (0.35)^{a}	2.68 (1.71)^{a}	3.46 (1.57)^{a}	21.4^{b}
	75	0.57 (0.33)^{ab}	0.68 (0.25)^{bc}	2.10 (1.99)^{b}	2.21 (1.38)^{b}	8.7^{b}
	125	0.46 (0.31)^{bc}	0.69 (0.45)^{c}	2.33 (1.88)^{ab}	2.43 (1.15)^{c}	8.2^{b}
III	0	0.83 (0.45)^{a}	0.99 (0.30)^{a}	3.23 (2.78)^{a}	3.97 (1.89)^{a}	82.0^{a}
	25	0.56 (0.33)^{b}	0.82 (0.56)^{b}	2.68 (2.28)^{b}	3.11 (1.83)^{a}	10.6^{b}
	75	0.45 (0.33)^{c}	0.61 (0.26)^{c}	1.65 (1.44)^{c}	2.00 (1.36)^{b}	2.2^{b}

Values within a period and column with identical superscripts were not significantly different according to the Kruskal-Wallis test (gonad weight and GSI; $P<0.05$) and the chi-square test (% of males shedding milt; $P<0.05$).

The mean body weight at the end of the experiment was not significantly different between hormone-treated groups and their controls ($P<0.05$) and ranged from 75.4 (period III, 75 ppm) to 87.3 (period I, control; Table 4). The relationships between the concentrations of 17β-E2 in the food and the dosage of hormone expressed as mg/kg body weight or as mg/kg body weight gain

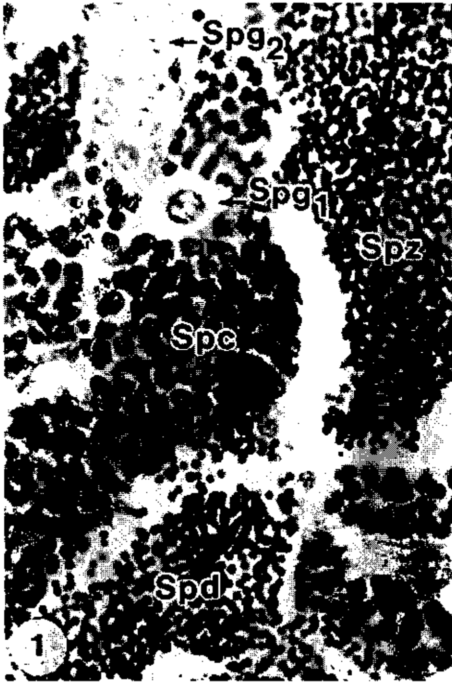
Figs. 1–6. Gonads of *C. carpio* aged 180 days, after various dietary treatments with 17α-MT or 17β-E2.

Fig. 1. Testis of a control group that contains immature and mature cysts. Spg_1=primary spermatogonium, Spg_2=secondary spermatogonia, Spc=spermatocyte, Spd-spermatids, Spz=spermatozoa (×1600).

Fig. 2. Ovary of a control group that contains lamellae with oogonia (Og), and previtellogenic oocytes (pOc) surrounded by a follicular layer (Fl) (×1600).

Fig. 3. Intersex gonad of a group treated with 17α-MT (50 ppm, 10–15 weeks after hatching) that contains testicular (T) and ovarian (O) tissue (×160).

Fig. 4. Intersex gonad of a group treated with 17α-MT (100 ppm, 6–11 weeks after hatching). Note the areas in which testicular and ovarian tissue are mixed. The oocytes (Oc) show advanced development, having circular rows of vacuoles (Vac) in their cytoplasm. The spermatogenic cysts are fused to form tubuli seminiferi (tS) (×400).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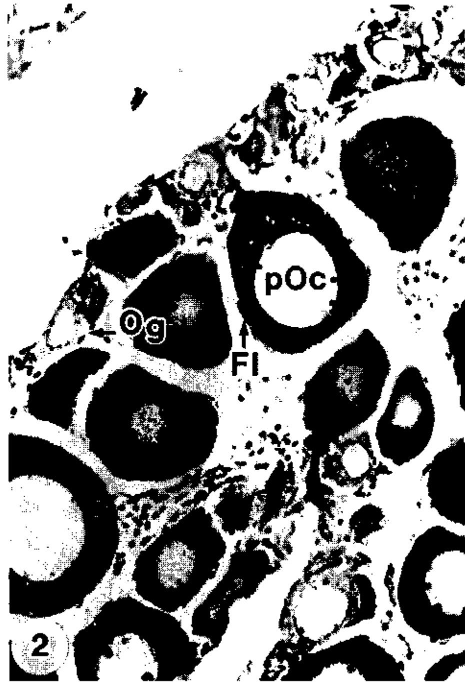
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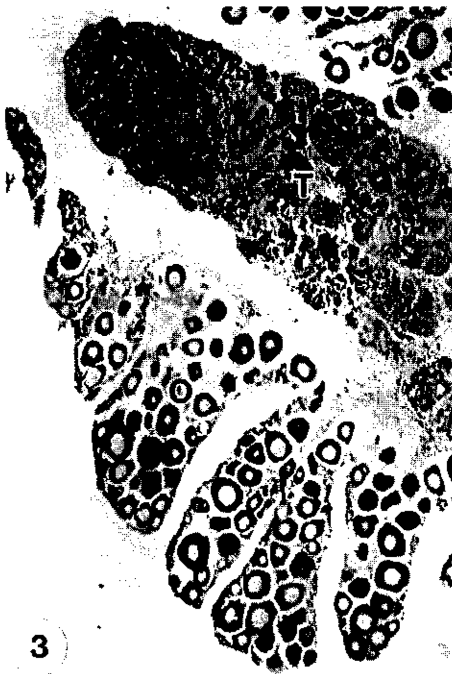
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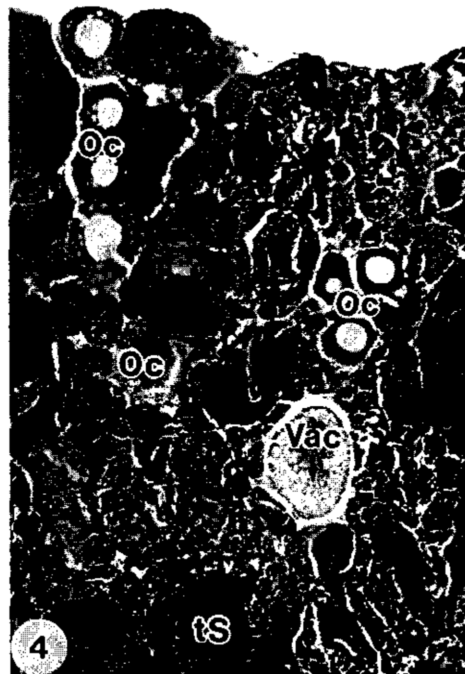
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(Table 4) are comparable with those observed in the first experiment with 17α-MT (Table 1). Due to high mortalities, dosages per kg body weight gain could not be calculated for groups fed 25 and 75 ppm during the first period (Table 4).

The frequencies of males were not significantly different between 17β-E2-treated groups and their controls and they were not significantly different from the expected 50% ratio ($P<0.05$; Table 5). Intersex gonads were found in hormone-treated fish and controls. Sterile gonads only occurred in hormone-treated fish.

Gonad weights and GSI of both females and males significantly decreased in each period with increasing concentrations of hormone in the food. This indicates that treatment with 17β-E2 has an inhibiting effect on gonadal development. Males and females of control groups had the highest gonad weights and GSI in each tested period. The number of males that produced milt after stripping was significantly lower ($P<0.05$; Table 6) in groups that were treated with 17β-E2 than in control groups and decreased in each test period with increasing concentrations of 17β-E2 in the food.

Histological examination

Gonads which could not be sexed macroscopically were either intersex or sterile. The latter category originated mainly from hormone-treated fish. There were no essential histological differences between gonads sampled in the first and second experiment.

Testes consist of irregularly shaped cysts, separated by interstitial tissue with blood vessels (Fig. 1). The cysts contain primary and secondary spermatogonia, spermatocytes, spermatids and spermatozoa. Mature cysts, filled with spermatozoa, are fused to form tubuli seminiferi (Fig. 1).

Ovaries (Fig. 2) consist of lamellae with oogonia and early prophase oocytes situated in the margins and previtellogenic oocytes located further inwards. The previtellogenic oocytes have a nucleus with one or more nucleoli. The latter type corresponds with stage II of the classification of Horvath (1985). All oocytes are surrounded by a thin follicular layer. Oocytes containing yolk granules were not found.

Intersex gonads contain clearly distinctive areas of testicular and ovarian tissue, separated by connective tissue (Fig. 3). The intersex gonads of hormone-treated fish often consist of areas in which testicular and ovarian tissue are mixed. The oocytes in these areas show advanced development, having circular rows of vacuoles in their cytoplasm (Fig. 4). These oocytes correspond to stage III/IV of Horvath (1985). In other intersex gonads the mixed areas of oocytes and spermatogenic cysts are fused, indicating that atresia has started (Fig. 5).

Sterile gonads are filiform and contain strands of connective tissue in which

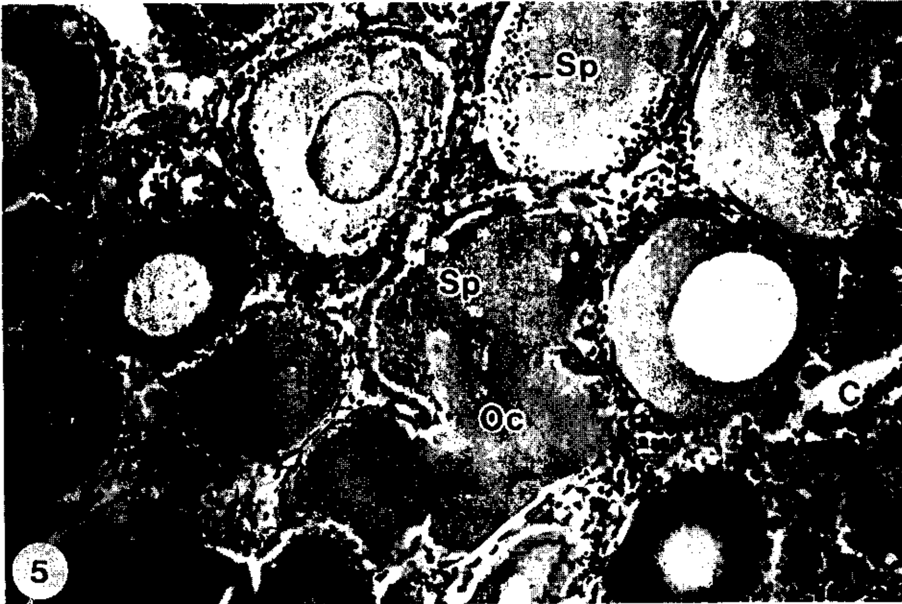

Fig. 5. Intersex gonad of a group treated with 17β-E2 (75 ppm, 6-11 weeks after hatching). Note the degenerating and fused oocytes (Oc), containing spermatids and spermatozoa (Sp) from neighbouring cysts (C) ($\times$1600).

Fig. 6. Sterile gonad of a group treated with 17α-MT (100 ppm, 3-8 weeks after hatching). Note the cluster of previtellogenic oocytes (pOc), embedded in connective tissue, and the absence of germ cells ($\times$400).

occasionally oocytes or spermatogonial cysts, but not germ cells, are found. The oocytes and spermatogonial cysts are often in a retarded stage of development (Fig. 6).

DISCUSSION

According to Yamamoto (1969), sex steroids should be administered during the entire period of gonadal differentiation in order to be effective. In carp, this period starts approximately 7–9 weeks after hatching, with a mitotic proliferation of germ cells, followed by a sexual differentiation of the primordial gonad. Testes and ovaries can be distinguished after histological examination 17 weeks after hatching (Parmentier and Timmermans, 1985).

In the present study with common carp, administration of 17α-MT in a concentration of 50 ppm in the food between 6 and 15 weeks after hatching yielded 92.7% male gonads. Earlier or later treatments gave less successful results. Between 3 and 8 weeks after hatching, treatment with 100 ppm of 17α-MT resulted in 93% sterile gonads and severe malformations of fish. These results are in strong contrast with those of Nagy et al. (1981) who found that male gonads could be induced in gynogenetic common carp by oral administration of 100 ppm 17α-MT during any period of 36 days between 8 and 80 days after hatching. The comparatively low number of sterile fish reported in their study could be attributed to the fact that only females were tested. However, the results of Nagy et al. (1981), in which the fish were fed ad libitum, are difficult to compare with our data since the dosage of hormone cannot be accurately expressed in mg/kg body weight or mg/kg body weight gain. This also holds for hormonal sex control studies in other fish species (see review by Hunter and Donaldson, 1983) in which dosages are expressed in mg/kg diet without mentioning the feeding levels applied.

In our experiments it is likely that the high feeding levels applied during 3 to 8 weeks after hatching resulted in an overdosage of 17α-MT with concomitant development of sterile gonads. It is therefore recommended to apply dosages which are related to body weight, taking into account growth increment (to be) realised over the experimental period, rather than feeding rations with fixed hormone concentrations (see Table 1).

Oral administration of 17β-E2 in the present study did not affect the sex ratio of common carp in any of the tested periods. It is not clear whether this result is typical for cyprinids since in the related goldfish (*Carassius auratus*) the less effective estrogen estrone induced female gonads (Yamamoto, 1975). In cichlids feminization with estrogens has not been successful in various tilapia species (Jensen and Shelton, 1979; Mair et al., 1986). In salmonids, oral administration of estrogens successfully produces females (*Salmo gairdneri*, Johnstone et al., 1978), but variable results have also been reported (Okada, 1973).

It is difficult to explain why sex induction in fish with androgens is, in gen-

eral, more successful than with estrogens. Failure to induce female gonads might be caused by a degradation of 17β-E2 in the liver, as suggested for *Salmo gairdneri* by Van den Hurk and Lambert (1982). According to Yamamoto (1969) and Hishida and Kawamoto (1970), 17β-E2 is less resistant to degradation in the digestive tract and liver than synthetic estrogens like ethynylestradiol. However, in our experiments with *C. carpio*, gonad weight and GSI of females and males decreased with increasing doses in each test period, indicating that the hormone had been effectively absorbed. Furthermore, the stripping response of males was significantly reduced after treatment with 17β-E2. Testosterone and estradiol (or their metabolites?) are indeed known to suppress gonadal development by inhibition of spermatogenesis and vitellogenesis (Billard et al., 1982; Lee et al., 1986).

A second explanation for the failure to induce female gonads with 17β-E2 in *C. carpio* can be found in the so-called dominant-neutral sex hypothesis. In juvenile rainbow trout, androgens and estrogens are known to exert a positive feedback on GTH syntheses (Van den Hurk, 1982; Goos et al., 1986). It is unknown whether GTH, during treatment with 17β-E2, in turn promotes androgen and estrogen synthesis in the differentiating gonad. Such a control might exist in tilapia. Hopkins et al. (1979) produced 90% females in *Tilapia aurea* (*Oreochromis aureus*) after treatment with ethynylestradiol in combination with metallibure, a pituituary blocker. Treatments with ethynylestradiol alone, or 17β-E2 alone or in combination with metallibure were not successful.

The evidence produced by Van den Hurk and Slof (1981), demonstrating the steroidogenic capabilities of rainbow trout testes but not ovaries at the time of sex differentiation, supports the dominant-neutral sex hypothesis. It suggests that in our experiments with common carp ovarian development could be interrupted or changed with 17α-MT, whereas testicular development with its own steroidogenic capability cannot be overruled by exogenous 17β-E2.

All control groups in the first and second experiments contained more males than females, which can be caused by small amounts of testosterone present in the pellets used (Sower and Iwamoto, 1985). However, it was recently found in our laboratory that certain crosses of inbred carp produce aberrant sex ratios. The possibility that these sex ratios are caused by the presence of homozygous recessive genes is under current investigation.

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Chapter 8

SEX CONTROL IN COMMON CARP (Cyprinus carpio L.)

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(Recent Advances in aquaculture IV, in press)

ABSTRACT

In this paper genetic sex determination and hormonal induced sex differentiation is discussed for common carp. The presence of a recessive mutant gene, which induces in homozygous condition maleness in gynogenetic offspring has been demonstrated.

INTRODUCTION

Sex differentiation in common carp is assumed to be regulated by genes, located on the so called sex chromosomes. Males are thought to be XY and females XX since gynogenetic reproduction of females produces exclusively female offspring (Nagy, 1986). Male sex inversion in gynogenetic females can be achieved by administration of androgens prior to phenotypic expression of gonadal sex (Nagy et al., 1981). Gonadal differentiation in this case takes place independent of the genotype (hormonal induced sex). Sex control in common carp by hormonal induction of sex is needed to establish reproducable inbred strains. In our laboratory we are interested to obtain inbred strains of carp for standardisation of endocrinological and immunological bio-assays. Another interest concerns selection on production characteristics such as late gonadal maturation. This can be achieved by crossing inbred strains to produce F_1 hybrids which display heterosis and reduced variation.
In this account we will first discuss the results of sex control by administration of sex steroids in our common carp broodstock. We will then analize the genetic basis of sex differentiation in some of the gynogenetic offsprings from this broodstock, and present evidence for the existence of a minor female sex determining gene. We will conclude by presenting the results of an experiment in which we investigated a possible interaction of hormonal induced sex with homozygosity or heterozygosity for this minor female sex determining gene, using homozygous and heterozygous clones of common carp.

SEX INVERSION WITH HORMONES

We have tested the effects of various dietary treatments with 17b-estradiol (E_2) and 17α-methyltestosteron (MT) on the gonadal development of common carp (Komen et al., 1989). Experimental diets containing various concentrations of MT or E_2 were prepared

by the alcohol evaporation method. For both hormones, three different periods of treatment were tested. A summary of the various treatments is given in table 1.

Table 1 Summary of treatments with MT and E_2. Diets containing various hormone concentrations were administered during 3 different periods after hatching. Alcohol treated diets containing no hormone were used as controls.

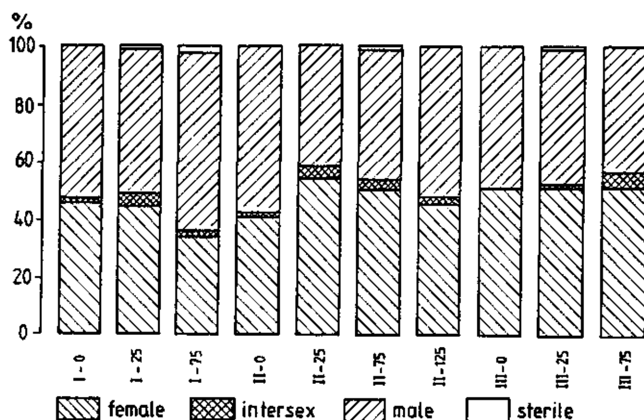
treatment	time after hatching (weeks)	concentrations of MT (ppm)				concentrations of E_2 (ppm)			
		0	50	100	150	0	25	75	125
I	3 - 8	-	+	+	-	+	+	+	-
II	6 - 11	+	+	+	+	+	+	+	+
III	10 - 15	-	+	+	-	+	+	+	-

Gonadal development of about 100 fish per group was assessed by macroscopical and histological examination 24 weeks after hatching, when the fish had reached a body weight of 80-100 gram. Gonads having both testicular and ovarian tissue were scored as intersex, while filliform gonads, in which occasionally oocytes or spermatogonial cysts were found, were scored as sterile. The results from these experiments can be summarized as follows (fig 1 and 2):

The frequencies of females in estradiol treated groups and their controls were not significantly different from the expected 50 % (fig.1). It is noteworthy that males receiving high hormone concentrations had reduced gonad weights and could not be stripped. This indicated that the hormone had effectively been absorbed. In contrast, the frequencies of males in groups treated with methyltestosterone during the second and third period were signifcantly higher than the expected 50 % (fig 2).

The optimum treatment for effective male sex inversion proved to be oral administration of 50 ppm MT in the food during 6 to 11 weeks after hatching (fig. 2: treatment II-50). Increasing the hormone concentration in the food apparently causes paradoxical feminization since the frequencies of intersexes and females slightly increased in these treatment groups. MT had a significant sterilizing effect on gonadal development when administered during the first period. Unexpectedly, the duplicate control groups in this

Figure 1 Frequencies of male, female, intersex and sterile gonads of common carp, after various dietary treatments with 17ß estradiol. The frequencies of males and and females were not significantly different between hormone treated groups and their controls, or the expected 50 % ratio, according to the Chi-square test (P<0.05)(see also table 1).

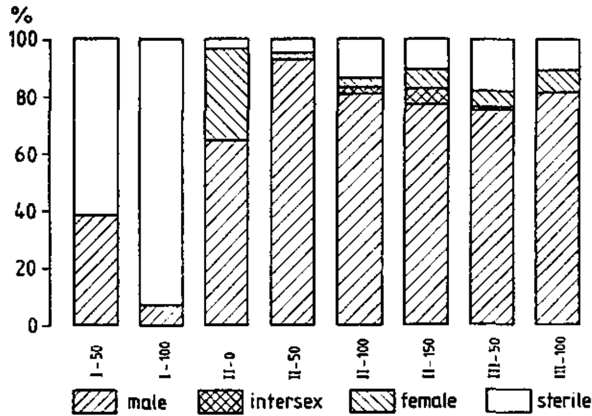


experiment (fig 2: treatment II-0) contained considerably more males (mean 64.4%) than females although this was not significantly different from the expected 1:1 sex ratio. This skewed sex ratio could not be attributed to failures in the experimental conditions. Such deviations from a 1:1 sex ratio, including all-female offspring from normal breedings, have occasionally been found in our laboratory during the last ten years.

SEX DIFFERENTIATION IN GYNOGENETIC CARP

In another series of experiments artificial gynogenesis was applied to compare the effects of inbreeding in heterozygous and homozygous gynogenetic common carp, and to produce homozygous clones (Komen et al., in prep.). In gynogenesis, eggs are fertilized with irradiated sperm and diploidy is restored by suppresion of either the second meiotic or first mitotic division. In the first case the degree of homozygozity in the offspring depends on the rate of crossing over between non-sister chromatids during the first meiotic division (retention of second polar body: 2PB-gynogenesis). In the second case fully homozygous diploid offspring are produced (endomitosis: EM-gynogenesis). The female used in this experiment was selected from one of our broodstocks, termed

Figure 2 Frequencies of male, female, intersex and sterile gonads ofcommon carp, after various dietary treatments with 17α methyltestosterone. Values are from pooled duplicates. The frequencies of males in all groups, except the controls, were significantly different from the expected 50 % ratio, according to the Chi-square test (P<0.05)(see also table 1).



WT. She was homozygous for a recessive gene for scalation (s/s; mirror carp), and normally pigmented but heterozygous for two recessive mutations which inhibit melanophore formation in homozygous condition (blond: $b_1,b_1/b_2,b_2$; Komen et al., submitted). The selected male from this broodstock was heterozygous scaled (+/s) and normally pigmented (+,+/+,+). Genetic inactivation of the sperm was achieved by irradiating milt, diluted 1:3 with 0.85% fysiological saline to a total volume of 10 ml, with U.V. (2200 J/m^2,min) for 60 min. A cold shock (0°C, 45 min; 1-2 min after fertilization) was used to produce 2PB-gynogenetic offspring (2PB group) while homozygous gynogenetic offspring (EM group) were produced by giving eggs a heat shock (40°C, 2 min; 30 min after fertilization (Komen et al.,1988; Komen et al.,1990 in press).

Control fry were produced by fertilizing eggs with non irradiated milt (CO group). The breedings and the resulting groups are summarized in Table 2.

None of the gynogenetic groups contained scaled individuals, indicating the absence of transmission of paternal genes, while 50 % of the fish in the CO group were scaled. Due to a high frequency of recombinantion between the two loci b_1 and b_2, and the centromere, only 6 % of blond fish were found in the 2PB group.

Table 2 Sex ratio's in normal and gynogenetic offspring of common carp.

Eggs from a female from the WT broodstock were fertilized with irradiated sperm from a WT male from this broodstock, and cold shocked or heat shocked to produce heterozygous (2PB) or homozygous (EM) offspring. Non irradiated milt from the WT male and a gynogenetic male GY was used to produce a control (CO) group and an all female offspring (WTxGY) respectively.

cross	offspring	sex ratio (%)			
		N	male	intersex	female
WT ♀ x WT ♂	CO-group	60	47.8	0.0	52.2
WT ♀ gynog.	2PB-group	60	3.3	3.3	93.4
WT ♀ gynog.	EM-group	60	21.6	25.1	53.4
WT ♀ x GY ♂	WTxGY-group	60	0.0	0.0	100.0

The homozygous nature of the EM group was confirmed by the presence of 23.8 % blond fish, which is not statistically different from the expected 25 %. Surprisingly, upon maturation of these gynogenetic offsprings, a high proportion of males and intersexes were found in the EM group (46.7 %) while in the 2PB group about 6.6 % males and intersexes occurred (Table 2). The control group on the other hand showed a normal sex ratio with nearly 50 % males.

Gynogenetic males had been found occasionally in other experiments among 2PB-gynogenetic offspring, but their origin was always unclear. We therefore crossed the WT female, who had been used to produce the aberrant 2PB and EM groups, with such an unrelated gynogenetic male GY. The offspring of this mating (WTxGY group; Table 2) contained only females. This indicated that neither the WT female nor the GY male carried a Y chromosome.In order to elucidate the cause of maleness in the EM and 2PB groups, we selected 3 females from the WTxGY group (nrs 1, 2 and 3) and crossed them with a selected male from the EM group. These crossings and the sexratio's in the resulting offsprings are summarized in Table 3. The 3 offsprings were raised to maturity and 6 months after hatching approximately 90 fish of each group were dissected and

macroscopically sexed. The offsprings of female 1 and 2 contained males plus intersexes, and females in an approximately equal ratio. The offspring of female 3 contained mainly females and a few intersexes and males.

Table 3 Sex ratio's in offspring from normal crosses of common carp.
Three females, selected from the (WT x GY)-group were each crossed with a homozygous gynogenetic male, selected from the EM-group.

cross	sex ratio (%)			
	N	male	intersex	female
(WTxGY) - ♀ 1 x EM ♂	90	43.3	13.4	43.3
(WTxGY) - ♀ 2 x EM ♂	99	27.3	33.3	38.4
(WTxGY) - ♀ 3 x EM ♂	89	1.1	4.5	94.4

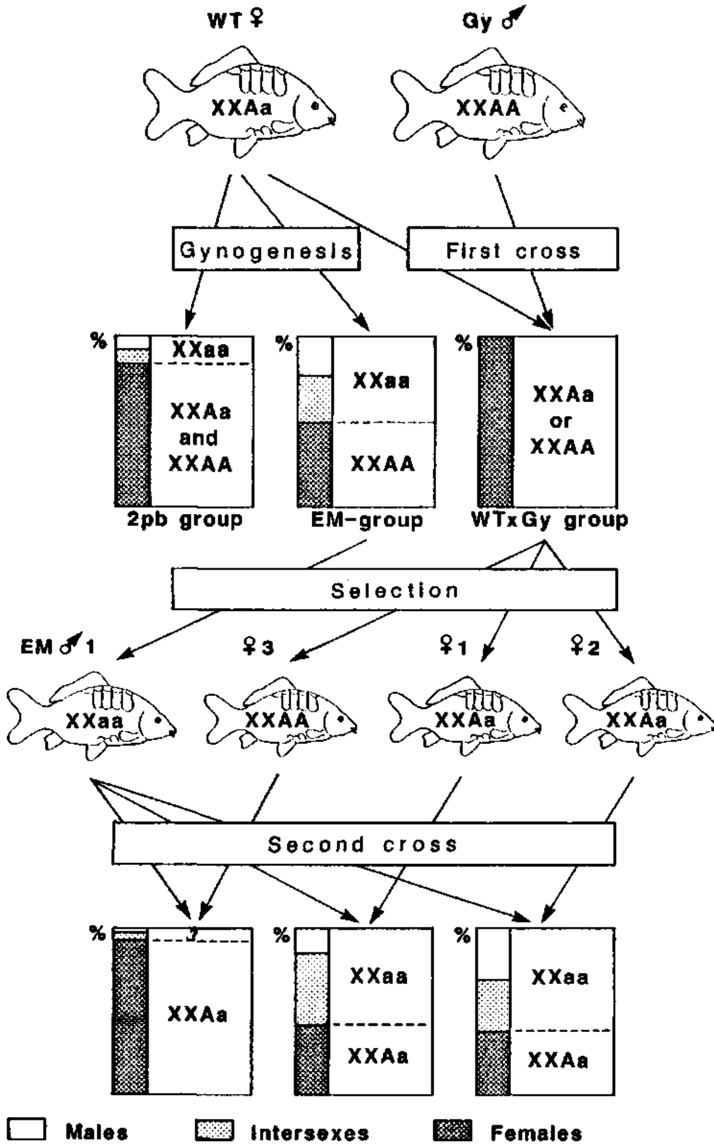
A MODEL FOR ATYPICAL SEX DETERMINATION IN GYNOGENETIC AND NORMAL COMMON CARP

The results (Table 2 and 3) can be explained by adopting the following model. Assume the presence of a minor female sex determining gene mas^{+}. The recessive allele (mutant gene) mas-1 in homozygous condition induces a male or intersex gonad in fish which are chromosomally XX (XX; mas-1/mas-1). A similar mutant gene, termed **male** sex determining gene, has been postulated by Kallmann (1984) to explain XX males in different Xiphophorus species.

The presence of nearly 50 % males and intersexes in the homozygous gynogenetic offspring (EM group) from the original WT female can then be explained by assuming her genotype to have been mas^{+}/mas-1, with mas^{+} and mas-1 seggregating in mas-1/mas-1 males and intersexes and mas^{+}/mas^{+} females (fig 3). The presence of 6.6 % males and intersexes (mas-1/mas-1 ?) in the 2PB group might indicate a high frequency of recombination between this locus and the centromere and thus a position distal on the chromosome arm (Thorgaard, 1983).

The majority of females in the 2PB group are therefore probably mas^{+}/mas-1. The GY

Figure 3 A model for atypical sex determination in gynogenetic common carp. Percentages indicated in the bars correspond with the data presented in table 2 and 3. For explanation see text.



male must be homozygous mas^{+}/mas^{+} since no males were found in the WTxGY-group. The actual genotype of GY is at present unknown, but preliminary results from backcrossings of (WTxGY)-females to this GY male have indicated the presence of two other mutant minor sex determining genes (Komen et al., in preparation).

If the WT female is assumed to be mas^{+}/mas-1 and the GY male mas^{+}/mas^{+}, then the genotypes of the females in the WTxGY group should be either mas^{+}/mas^{+} or mas^{+}/mas-1. This was corroborated by the results from the second series of crosses (see table 2 and fig 3). The WTxGY females 1 and 2 were apparently mas^{+}/mas-1 since the cross with the selected EM male (mas-1/mas-1) produced females (mas^{+}/mas-1) and males and intersexes (mas-1/mas-1) in approximately equal ratio's. The WTxGY female 3 must have been mas^{+}/mas^{+} since she produced mainly mas^{+}/mas-1 female offspring after crossing with the EM male mas-1/mas-1.

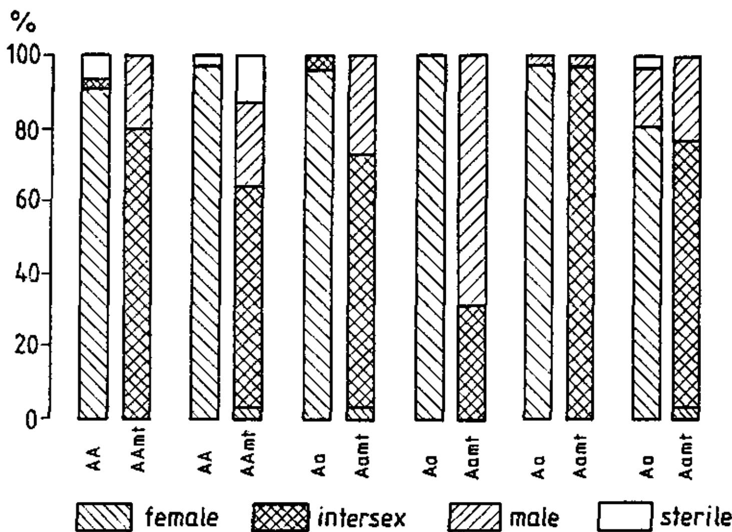
We believe that the non-gynogenetic fish used in the first series of experiments with MT were the offspring of a cross XX;mas^{+}/mas-1 x X/Y;mas^{+}/mas-1, and that the skewed sex ratio (64.4 % males) in the control group (fig 2, treatment II-0) was due to the presence of XX;mas-1/mas-1 males (Table 4).

Table 4 Punnet square for possible genotypes in an offspring from a cross XX; mas^{+}/mas-1 x XY;mas^{+}/mas-1. The frequency of males (bold type) in this offspring will be 5/8 or 62.5 %.

	X,mas^{+}	X, mas-1
X,mas^{+}	XX;mas^{+}/mas^{+}	XX;mas^{+}/mas-1
X,mas-1	XX;mas^{+}/mas-1	XX;mas-1/mas-1
Y,mas^{+}	XY;mas^{+}/mas^{+}	XY;mas^{+}/mas-1
Y,mas-1	XY;mas^{+}/mas-1	XY;mas-1/mas-1

In order to investigate a possible interaction between the effects of hormone treatment and these sex determining genotypes, we performed an experiment in which carp clones with genotypes XX;mas$^+$/mas$^+$ and carp clones with genotypes XX;mas$^+$/mas-1 were treated with 17a methyltestosterone to induce male sex inversion. Per clone about 50 fish were used for the treatment while another 50 fish were used as untreated controls. The treatment used (50 ppm, 6-11 weeks after hatching) was the optimum treatment for male sex inversion, found in the first series of experiments. The results are summarized in fig 4.

Figure 4 Frequencies of male, female, intersex and sterile gonads invarious clones of common carp, after dietary treatment with 17α- MT. Clones which were homozygous or heterozygous for a minor female sex determining gene mas$^+$ (A), were treated with 50 ppm 17α MT, 6- 11 weeks after hatching. Untreated fish from each clone served as a control on normal sex differentiation.



All hormone treated groups except one contained a very high percentage of intersexes (60-90 %) in comparison to the previous experiment (compare fig 2 and 4). Only one hormone treated clone contained significantly more males than intersexes (69 % vs 20.3%). The controls, as could be expected, contained mainly females. The percentages of male sex inverted fish could not be correlated with homo- or heterozygosity for the sex determining gene mas^{+}. The high percentages of intersex gonads occuring in all hormone treated groups (exept one) indicate that other (regulatory?) genes in these various clones more or less determine the succes of hormonal induced sex inversion.

DISCUSSION

According to Yamamoto (1969) sex steroids should be administered during the period of gonadal differentiation in order to be effective. Despite this general rule, however, little is known as to the exact mechanism in which exogenous androgens and estrogens exert their influence on the developing gonad. Male sex inversion can succesfully be induced by administration of various androgens in salmonids (Johnstone et al., 1978), tilapias (Rothbard et al.,1983) and in carps (Nagy et al., 1981, Komen et al., 1989).
On the other hand, female sex inversion by the use of various estrogens is often only partially succesful or not succesful at all in tilapias and carps. In the first series of experiments 17ß estradiol did not affect the sex ratio in any of the tested dosages or periods. A possible explanation for these findings can be found in the so called induced-neutral sex hypothesis. In this hypothesis, maleness can be induced in the neutral female sex but not vice versa. In rainbow trout the steroidogenic capability of testes but not ovaries were demonstrated at the time of sex differentiation (Van den Hurk and Slof, 1981). This could explain in our experiments with common carp why ovarian development can be changed by MT while testicular development with its own steroidogenic capacity cannot be overruled by exogenous E2. It should be noted however that the succes of a hormonal treatment often seems to depend on the species used. Various closely related tilapia species show differences in sensitivity to treatment with estrogens. Estradiol treatment was ineffective in carps while in the closely related goldfish the less effective estrogen estrone induced female gonads (Yamamoto, 1975). Similarly , Nagy et al. (1981) obtained succesfull male sex inversion in Hungarian carps with 100 ppm MT administered during any period of 36 days between 1 and 12 weeks after hatching,

while our results with Dutch carps revealed a much shorter sensitive period of 6 to 11 weeks after hatching. These findings indicate that the genetic background of a carp species is an important factor in hormonal induced sex inversion. The results obtained in the third series of experiments furthermore show that even individual genotypes (e.g. of each of the clones tested) more or less determine the succes of hormonal induced male sex inversion.

The presence of a minor female sex determining gene of which the recessive (mutant) allele in homozygous condition induces a male or intersex gonad in gynogenetic offspring has, as far as we know, never been observed in fish. The discovery of this minor female sex determining gene indicate a complex system of genes apart from those located on X and Y involved in sex determination in common carp. With respect to their role in sex differentiation it is attractive to assume that they code for specific enzymes which are involved in sex steroid synthesis or for specific steroid receptors.

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Summary

The absence of well defined inbred lines is an important problem associated with scientific research on fish. Inbred lines can be produced by conventional full-sib mating, but at least 10-15 generations are needed to produce homozygous inbred lines. Using common carp, which reach maturity at 1.5 years, this would last some 15-30 years. Nowadays experimental fishes are usually obtained from commercial fish farms, or bred in the laboratory using a limited number of broodstock fish. In both cases the genetic background and the degree of inbreeding of the experimental animal is unknown.
In consequence the results from various laboratories are difficult to compare. Bioassays often show a large variation in the experimental results and a relative low reproducability. Moreover, large numbers of fish are needed to obtain statistically significant results. In order to solve these problems this research project was started with the aim to develop homozygous inbred lines of fish by gynogenetic breeding. Furthermore, in our university there was a high need for inbred lines with specific (mutant) genotypes, which could be used in the ongoing research on the immune system and sex determination of common carp.

In gynogenesis, eggs are fertilized with genetically inactivated sperm. The resulting haploid embryo can be made diploid by inhibition of the second meiotic division (retention of the second polar body or 2PB method), or by inhibition of the first mitotic division (endomitosis or EM method). In the first case the gynogenetic offspring will be partly heterozygous due to recombination during the preceeding meiotic prophase. In the second case the haploid genome of the embryo is duplicated while the first cell division is prevented. The resulting diploid offspring will be fully homozygous.
In a first series of experiments (chapter 3) the optimal conditions for irradiation and dilution of milt, and for administration of a temperature shock to inhibit the second meiotic division, were investigated. Milt was irradiated with U.V. light (235.7 nm). Dilution (in physiological saline) and irradiation duration were important parameters for the survival of spermatozoa. Sperm, diluted 1:3, could be irradiated for 60 minutes (2200 J/m2,min) without loss of fertilization capacity. This fertilization capacity was considerably reduced when higher dilutions were used, while a shorter irradiation period failed to inactivate all spermatozoa.

The effectiveness of genetic inactivation was checked by using sperm from scaled males (a dominant trait) and eggs from scattered females (recessive trait). Gynogenetic offspring turned out to be all scattered. Inhibition of the second meiotic division was achieved by administering eggs, fertilized with genetically inactivated sperm, a temperature shock at various moments after fertilization. Consistent yields of 25- 50 % viable fry were obtained when eggs were cold shocked (0°C) for 45 minutes, 1-2 or 7-9 minutes after fertilization (at 24 °C). This bimodal response was typical for common carp, but essentially different from other investigations on common carp gynogenesis, where lower incubation temperatures and degumming of egg was practised.
In a second series of experiments (chapter 4) the optimal conditions for inhibition of the first mitotic division were investigated. The occurrence of metaphase of the first mitotic division was histologically determined. Consistent yields of 5 - 15 % viable fry were obtained when eggs were heat shocked at 40 °C for 2 minutes, 28-30 minutes after fertilization (i.e. at metaphase). Accurate timing of the heat shock, as well as the heat shock temperature and duration, were critical in obtaining an optimal yield of diploid fry. The homozygous nature of the gynogenetic fry was demonstrated by the Mendelian segregation patterns of two recessive colour mutations (chapter 4).

An important aspect of the described gynogenetic breeding techniques is the effect of the expected homozygosity in a first generation of gynogenetic offspring. In order to investigate this effect, we compared homozygous carps (EM method) with heterozyous gynogenetic carps (2PB method) and a group obtained by full-sib mating (chapter 5). The three groups were all obtained from the same mother, and allowed a comparison of the effects of increasing levels of homozygosity. Skin grafts were exchanged between animals of the same group and between animals of different groups. Skin allografts exchanged among heterozygous gynogenetic carp exhibited prolonged survival. Furthermore a strong histocompatibility (H) locus was seen to segregate in this group. In contrast skin allografts exchanged among homozygous gynogenetic siblings or among normal full-sibs were all rejected in an acute manner, with homozygous fish showing the most vigorous allograft reactions. These findings were explained by assuming that acute allograft reactions were the result of a single strong H-locus disparity, or of a multiple minor H-loci barrier which mimics a strong H-locus effect (chapter 5).

In a follow-up experiment (chapter 6) the effects of increasing levels of homozygosity on sex, gonad development and fertility of carps from these three groups were compared. Surprisingly nearly 50 % males and fishes with intersex gonads were found in the EM group while males were absent in the 2PB group. This excluded a possible contamination with non-irradiated (non-inactivated) sperm. Inbreeding significantly increased the mean gonad weight as well as the variation in gonad weights. Full sib (FS) and heterozygous gynogenetic offspring (2PB) were normal in gonad development, but gonads from homozygous gynogenetic (EM) carp were often retarded in vitellogenesis. The ovulation response was significantly reduced with increasing levels of inbreeding. Eggs from ovulated females of the FS, 2PB and EM groups were fertilized with milt from males of the FS and EM groups. Yields of normal fry were reduced in crosses involving FS and 2PB eggs when compared to crosses with EM eggs or milt. This indicated that homozygous fish were essentially free of recessive lethal genes affecting embryo survival (chapter 6).

New inbred lines were produced using a combination of both gynogenetic techniques. Homozygous inbred strains were produced by gynogenetic reproduction (2PB method) of homozygous gynogenetic (EM) females. F_1 hybrid strains were produced by crossing homozygous females with homozygous gynogenetic male siblings. The clonal nature of these strains was unequivocally demonstrated by reciprocally exchanged skin allografts. All grafts exchanged among members of the same strain were permanently accepted. Likewise grafts from homozygous strain members were accepted by fish from the related half-sib F1-hybrid strains, while the reverse grafts were rejected. These results provided evidence for the idea that in carp, as in other vertebrates studied so far, histocompatibility genes exist as major and minor loci which are codominantly expressed (chapter 5).

The inbred strains and F1 hybrids were comparable in body weight and gonad development (chapter 6), but the F1 hybrids showed a much lower variation in body weight and gonad development. In contrast the phenotypic variation was considerably enlarged in the homozygous inbred strains. This phenomenon is well known in inbred strains of mice and rats, and are generally attributed to developmental instability. The F1 hybrids are therefore more suited for use in bioassay's, especially since they might

possess an increased viability.

One of the advantages of the described gynogenetic inbreeding system is that selection of the most interesting and viable genotypes is required only in the first generation. The selected females can be propagated to produce inbred strains are identical to their parents in overall performance. However, in order to obtain males within a gynogenetic inbred line, some females should be sex-inversed by hormonal treatment. Therefore juvenile, non-inbred carps were treated with various doses of orally administrated 17α methyltestosterone during different periods after hatching. The treatment periods were 3-8 weeks, 6-11 weeks and 10-15 weeks after hatching. The tested hormone concentrations in the food were 50 and 100 ppm, while a dose of 150 ppm was also applied during 6-11 weeks after hatching. The gonads were inspected at 6 months after hatching. Administration of 50 ppm 17α-MT in the food between 6 and 11 weeks after hatching resulted in 92,7% males. Earlier treatments with 17α-MT in concentrations of 50 and 100 ppm of hormone in the food resulted in high percentages of sterile fish while later treatments produced a high percentage of intersex gonads (chapter 7). Surprisingly a similar experiment using 17ß estradiol failed to induce female gonads in any of the periods tested and irrespective of the concentrations of hormone used.

The optimal treatment with methyltestosterone was used to induce sex-inversion in the produced homozygous inbred strains and F1 hybrids (chapter 8). The untreated groups contained females and a single fish with intersex gonads. In the treated groups however, mainly intersex gonads were observed. Only one F1 hybrid group contained significantly more males (60 %) than animals with intersex gonads. These results can only be explained by assuming that the success of hormone induced sex inversion is genetically determined.

Maleness in common carp is thought to be determined by dominant sex determining genes, since heterozygous gynogenetic offspring were all female. However, in some homozygous gynogenetic offspring nearly 50 % males and intersexes were found. It was therefore suggested that maleness in these groups might be caused by recessive mutations in sex determining genes. The mother of one offspring group, probably heterozygous for a putative mutation, was crossed with an unrelated gynogenetic male from another experimental group. The offspring of this cross was exclusively female, but crosses of these females with gynogenetic males contained again 50 % males and

intersexes. It was concluded that these males and intersexes were homozygous for a recessive mutant sex determining gene termed mas-1. To our knowledge such mutations have not been described in fish before (chapter 8).

In conclusion, it can be stated that gynogenesis is a very successful and rapid method for the production of homozygous inbred lines of the common carp, *Cyprinus carpio*. Such inbred lines have until now only been produced in two small aquarium fish species, zebrafish (*Brachydanio rerio*), and medaka (*Oryzias latipes*). Our new inbred lines of common carp will be very important for future scientific research. The use of F1 hybrids in endocrinological and immunological bioassays will result in an increased standardisation and thus in a reduction of the number of experimental animals needed. Perhaps the inbred lines can also provide an alternative for the use of other experimental vertebrate animals. The present study also demonstrated the possibilities of gynogenetic breeding in unravelling complex biological processes as graft rejection and sex determination. Moreover, the rapid isolation of specific mutants with an abnormal development may offer important possibilities for future research.

Samenvatting

Een belangrijk probleem bij het gebruik van vissen in wetenschappelijk onderzoek is het ontbreken van genetisch gedefinieerde inteeltlijnen. Bij conventionele inteelt zijn in het algemeen ongeveer 10-15 generaties van "full-sib" (broer-zus) paring benodigd om bij benadering homozygote lijnen te verkrijgen. Met een generatie-duur van 1,5-2 jaar bij de karper duurt dit zo'n 15-30 jaar. Voor experimenten worden vaak vissen uit commerciële viskwekerijen betrokken of wordt er gewerkt met vissen welke in het laboratorium uit een beperkte groep ouderdieren zijn verkregen. In beide gevallen is er sprake van een heterogene groep proefdieren met onbekende genetische achtergrond, terwijl er tevens sprake kan zijn van een aanzienlijke mate van onbedoelde inteelt. Hierdoor zijn onderzoeksresultaten onderling of afkomstig van verschillende laboratoria soms moeilijk vergelijkbaar, en vertonen bijvoorbeeld bio-assays een zeer grote spreiding in de gemeten respons terwijl de herhaalbaarheid gering is. Een van de gevolgen is het gebruik van aanzienlijke aantallen proefdieren om statistisch verantwoorde conclusies te kunnen trekken. Om deze problemen te ondervangen werd een onderzoek gestart met als doel om via een kunstmatige vorm van parthenogenese (gynogenese) ingeteelde lijnen bij de karper te produceren. Het onderzoek moest tevens een eerste aanzet vormen tot de ontwikkeling van inteeltlijnen met zeer specifieke (mutante) genotypen ten behoeve van bestaand onderzoek aan het immuunsysteem en de geslachtsdifferentiatie bij de karper.

Bij gynogenese worden eieren "bevrucht" met genetisch inactief sperma. Het in potentie haploide embryo kan diploid worden gemaakt door blokkering van de tweede meiotische deling (vasthouden van het tweede poollichaampje of 2PB-methode) of de eerste mitotische deling (endomitose of EM-methode). In het eerste geval zijn de gynogenetische nakomelingen niet volledig homozygoot door het optreden van recombinatie tijdens de voorafgaande profase van de meiose. In het tweede geval wordt het haploide genoom van het embryo verdubbeld zonder dat de eerste celdeling plaatsvindt. De resulterende diploide nakomelingen zijn in principe volledig homozygoot.

In een eerste serie experimenten (hoofdstuk 3) werden de optimale condities van bestraling en verdunning van het sperma, en toediening van de temperatuurschok aan

de eieren onderzocht. Tevens werd gekeken wat de invloed was van variaties in incubatie condities van de eieren op de opbrengst aan 2PB gynogenetische larven. Voor de bestraling werd op grond van de literatuur gekozen voor U.V.-C licht (253.7 nm). Uit de resultaten bleek dat verdunning (in een fysiologische zout oplossing) alsmede bestralingsduur van invloed waren op de overleving van het sperma. Bij een optimale verdunning van 1:3 was het sperma na 60 minuten bestraling (2200 J/m2, min) genetisch geinactiveerd zonder verlaging van de bevruchtings-capaciteit. Bij een sterkere verdunning werd de bevruchtingscapaciteit aanzienlijk gereduceerd, terwijl bij een kortere bestralingsduur niet al het sperma genetisch geinactiveerd werd. Ter controle op onvolledige inactivatie werd sperma van geschubde mannetjes (dominant kenmerk) en eieren van ongeschubde vrouwtjes (recessief kenmerk) gebruikt. Gynogenetische nakomelingen bleken inderdaad uitsluitend ongeschubd te zijn. Blokkering van de tweede meiotische deling geschiedde door eieren, na bevruchting met bestraald sperma, gedurende een korte tijdsfase na bevruchting, een koudeschok toe te dienen. De opbrengst aan vitale larven bedroeg doorgaans 25-50 %, wanneer de eieren 1-2 of 7-9 minuten na bevruchting (bij 24°C) gedurende 45 minuten in ijswater (0°C) werden gedompeld. Deze tweetoppige respons bleek typisch voor karper, maar te verschillen van andere onderzoekingen waarbij lagere incubatietemperaturen en ontkleving van de eieren werden toegepast.

In een volgende serie experimenten (hoofdstuk 4) werden de optimale condities voor verstoring van de eerste mitose (EM-methode) onderzocht. Het tijdstip waarop de metafase optreedt werd langs histologische weg vastgesteld. Vervolgens vond blokkering van de mitose plaats door eieren 28-30 minuten na bevruchting (d.w.z. tijdens de metafase) een hitteschok (van 24 °C naar 40°C gedurende 2 minuten) toe te dienen. De opbrengst aan vitale homozygote larven bedraagt onder deze condities slechts 5-15% van het aantal bevruchte eieren. Acurate 'timing' van de hitteschok alsmede de hitteschokduur en -temperatuur bleken binnen zeer nauwe grenzen bepalend voor de overleving. De homozygotie van de nakomelingen werd aangetoond door gebruikmaking van vrouwelijke ouderdieren welke heterozygoot waren voor twee recessive kleurmutaties. De pigmentatie typen werden in strict Mendelse verhoudingen in de gynogenetische nakomelingen teruggevonden hetgeen duidde op een afwezigheid van heterozygoten (hoofdstuk 4).

Een belangrijk aspect van de beschreven gynogenetische inteelttechnieken is het gevolg van de te verwachten homozygotie in een eerste gynogenetische generatie. Om deze te onderzoeken werden homozygote karpers (EM methode) vergeleken met karpers, verkregen door normale broer-zus paring (Full-Sib = FS) en 2PB-gynogenese (2PB). Deze karpers hadden allen dezelfde moeder. Hierdoor was het in principe mogelijk de optredende homozygotie te kwantificeren. Huidtransplantaten werden uitgewisseld tussen dieren van de dezelfde groep en tussen dieren uit verschillende groepen. Het bleek dat alle huidjes uitgewisseld tussen dieren binnen de FS of EM groepen snel werden afgestoten. Daarentegen vertoonden de afstotingstijden van huidjes uitgewisseld tussen 2PB dieren een zeer grote spreiding. Bovendien was de invloed van een sterk histocompatibiliteits- of transplantatie-locus, naast meerdere zwakke loci, in deze groep aantoonbaar. Deze resultaten duidden op een grote mate van heterozygotie en dus isogenie voor vele histocompatibiliteitsgenen in de 2PB nakomelingsschap. Daarentegen waren de vissen in de EM groep genetisch blijkbaar zeer verschillend, wat op grond van volledige homozygotie voor vele loci op verschillende chromosomen paren ook verwacht werd (hoofdstuk 5).

In een vervolg-experiment (hoofdstuk 6) werden van een aantal vissen uit de drie genoemde groepen diverse kenmerken met betrekking tot de voortplanting gemeten. Verassend was het voorkomen van bijna 50 % dieren met mannelijke of intersex gonaden in de EM groep. Mannetjes waren echter afwezig in de gynogenetische 2PB groep, waardoor een contaminatie met genetisch niet geinactiveerd sperma kon worden uitgesloten. De effecten van inteelt manifesteerden zich verder als een sterk toegenomen variatie in lichaams-, ovarium- en eigewicht met toenemende mate van homozygotie. De inteeltdepressie kwam vooral in de EM groep tot uiting als een stijging in het gemiddeld ovariumgewicht, een daling van het gemiddelde eigewicht, en een sterk verminderde ovulatierespons. In de FS-groep waren alle vrouwelijke dieren na hormonale inductie afstrijkbaar, terwijl bij de 2PB-groep 65% en bij de EM-groep slechts 20% afstrijkbaar was. De eieren, afkomstig van geovuleerde vrouwtjes uit de FS, 2PB en EM groep, werden vervolgens bevrucht met sperma van mannetjes uit de FS groep. Tevens werden eieren van FS-vrouwtjes bevrucht met sperma van EM-mannetjes. Kruisingen tussen FS en EM dieren gaven significant minder misvormde larven en meer normale larven dan de andere kruisingen. Dit betekende dat gameten van EM-homozygote vrouwtjes en

mannetjes vrij waren van recessief lethale genen welke embryonale sterfte kunnen veroorzaken (hoofstuk 6).

Door toepassing van een combinatie van beide gynogenese technieken werden ingeteelde lijnen geproduceerd. Homozygote vissen, verkregen volgens de EM-methode, werden nogmaals voortgeplant volgens de 2PB methode. Deze gynogenetische nakomelingen zijn homozygoot en genetisch identiek zodat van een inteeltlijn gesproken kan worden. Daarnaast werden homozygote vrouwtjes gekruisd met homozygote EM mannetjes. Deze kruisingen tussen homozygote vissen zijn ook genetisch identiek maar heterozygoot. Deze groepen kunnen als F1 hybriden worden beschouwd. Het klonale karakter van de inteeltlijnen en F1 hybriden werd aangetoond door huid transplantaties uit te voeren. Huidjes, uitgewisseld tussen leden van dezelfde groep, werden zonder meer geaccepteerd. Huidjes, uitgewisseld tussen leden van verschillende homozygote inteeltlijnen, werden snel afgestoten terwijl huidjes van homozygote vissen werden geaccepteerd door F1 hybriden indien deze de moeder met de homozygote donoren gemeenschappelijk hadden. Omgekeerd werden de huidjes afgestoten. Deze resultaten toonden aan dat bij de karper, evenals bij zoogdieren, histocompatibiliteitsgenen codominant tot expressie komen (hoofdstuk 5).

Met betrekking tot het lichaamsgewicht en de gonadenontwikkeling waren er grote verschillen tussen de inteeltlijnen en de F1 hybriden (hoofdstuk 6). De F1 hybriden hadden op dezelfde leeftijd een hoger gemiddeld lichaams- en gonadegewicht dan de homozygote inteeltlijnen, terwijl de spreiding aanzienlijk gereduceerd was. Omgekeerd vertoonden de homozygote inteeltlijnen een sterk vergrote individuele spreiding in gewicht en gonade-ontwikkeling. Dit fenomeen komt ook voor bij inteeltlijnen van muizen en ratten, en wordt toegeschreven aan ontwikkelingsinstabiliteit. Het feit dat dit verschijnsel ook bij karpers optreedt doet vermoeden dat een instabiele ontwikkeling een direkt gevolg is van de volledige homozygotie van het dier. Voor het gebruik in bioassays lijken derhalve kruisingen tussen homozygote dieren meer perspectieven te bieden, mede ook vanwege de verbeterde larvale overleving en de te verwachten hoge vitaliteit.

Het voordeel van het hier beschreven twee generaties gynogenetische inteeltmodel is,

dat slechts één keer (in de eerste generatie) op de meest vitale maar ook meest interessante genotypen geselecteerd hoeft te worden. De geselecteerde ouderdieren kunnen gynogenetisch voortgeplant worden om inteeltlijnen te produceren welke qua prestatie niet afwijken van de uitgangspopulatie.
Om echter mannelijke vissen binnen de inteeltlijnen te verkrijgen dient een deel van de gynogenetische dieren hormonaal geinduceerde geslachtsomkeer te ondergaan. Teneinde hiervoor een optimale procedure te ontwikkelen werden juveniele karpers, afkomstig uit niet ingeteelde lijnen, op verschillende leeftijden gedurende 5 weken behandeld met via het voer toegediend 17α-methyltestosteron. De geteste perioden waren 3-8 weken na uitkomen van het ei, 6-11 weken na uitkomen, en 10-15 weken na uitkomen. De geteste concentraties hormoon in het voer waren respectievelijk 50, en 100 ppm. Voor de periode 6-11 weken werd tevens een concentratie van 150 ppm getest. Bij inspectie van de gonaden na 6 maanden bleek dat een vroege behandeling tussen 3 en 8 weken resulteerde in een hoog percentage sterile vissen. Een late behandeling, 10-15 weken na uitkomen, resulteerde in een hoog percentage intersex gonaden. De beste resultaten, 92.7 % mannelijke gonaden, werden verkregen indien karpers gedurende 6-11 weken na uitkomen 50 ppm 17α methyltestosteron in het voer kregen toegediend. Interessant was dat een identieke proefopzet, uitgevoerd met 17ß oestradiol in de concentraties 25 ,75 en 125 ppm, geen enkel feminiserend effect sorteerde (hoofdstuk 7).

De optimale behandelings methode met methyltestosteron werd vervolgens toegepast op de geproduceerde inteeltlijnen en F1 hybriden (hoofdstuk 8). De onbehandelde inteeltlijnen en F1 hybriden bevatten na 6 maanden uitsluitend vrouwelijke vissen. Bij de behandelde groepen werden echter voornamelijk intersex gonaden aangetroffen. Slechts één F1 hybride groep bevatte meer dan 60 % mannetjes, wat duidde op een geslaagde behandeling. Deze resultaten worden vooralsnog verklaard door aan te nemen dat het succes van de hormonale geslachtsinductie mede genetisch bepaald is. Het mannelijke geslacht bij de karper wordt verondersteld te zijn bepaald door dominante geslachtsdeterminerende genen. Het feit dat de onbehandelde gynogenetische groepen normaal uitsluitend vrouwelijk zijn ondersteunt deze aanname. Het optreden van mannetjes en dieren met intersexgonaden in sommige gynogenetische groepen duidde er echter op dat mannelijkheid in deze groepen mogelijk veroorzaakt werd door mutaties in andere geslachtsdeterminerende genen. Onder de aanname dat de moeder van de

gynogenetische mannetjes heterozygoot was voor een dergelijke mutatie, werd zij gekruisd met een niet verwant gynogenetisch mannetje uit een andere proefgroep. De nakomelingen waren wederom allen vrouwelijk maar kruisingen van deze dochters met hun oorspronkelijke gynogenetische broertjes leverde weer 50 % mannetjes en dieren met intersexgonaden op. Dit bevestigde dat mannelijkheid in de bewuste gynogenetische groepen veroorzaakt werd door een recessieve mutatie, genaamd mas-1. Voor zover ons bekend zijn dergelijke mutaties nog niet eerder bij vissen beschreven (hoofdstuk 8). Concluderend kan worden gesteld dat gynogenese een zeer snelle en effectieve methode is voor het produceren van homozygote inteeltlijnen bij de karper, *Cyprinus carpio*. Dergelijke gynogenetische inteeltlijnen zijn tot op heden alleen bij twee aquariumvissen, het zebravisje (*Brachydanio rerio*) en het rijstvisje (*Oryzias latipes*), geproduceerd.

De nieuwe karper inteeltlijnen zijn van groot belang voor het wetenschappelijk onderzoek aan vissen. Het gebruik van geselecteerde F1 hybriden in endocrinologische en immunologische bio-assay's zal tot een verhoogde standaardisatie leiden met een daaraan gekoppelde reductie van het aantal benodigde proefdieren. De inteeltlijnen kunnen bovendien mogelijk een alternatief bieden voor het gebruik van warmbloedige vertebraten, zoals knaagdieren.

Uit het onderzoek is tevens gebleken dat gynogenese een efficiënte manier kan zijn om de genetische achtergrond van complexe biologische processen op te helderen. Met name de snelle isolatie van mutaties, welke de normale ontwikkeling van het individu verstoren, biedt hierbij interessante mogelijkheden voor de toekomst. De beschrijving van de genetische principes van de transplantaatafstoting en de mutaties in het geslachts-bepalend systeem bij de karper zijn hiervan een goede illustratie.

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Curriculum vitae

Johannes Komen werd op 28 oktober 1957 te Amsterdam geboren. Hij behaalde in 1976 het Atheneum-B diploma aan de scholengemeenschap Noord te Amsterdam. In 1978 begon hij met zijn studie Biologie aan de Vrije Universiteit Amsterdam. In juli 1982 behaalde hij het kandidaatsdiploma in de richting Biologie - wiskunde. In januari 1983 begon hij met een doctoraalstudie Visteelt aan de Landbouw universiteit Wageningen. In augustus 1985 behaalde hij cum laude het doctoraalexamen, met als hoofdvakken Visteelt en Waterzuivering, en als bijvak Erfelijkheidsleer. Op 1 november 1985 trad hij als wetenschappelijk assistent in dienst van de Vakgroepen Visteelt en Visserij en Experimentele Diermorfologie en Celbiologie. Hij verrichtte daar gedurende 4 jaar het hier beschreven onderzoek. Momenteel is hij tijdelijk werkzaam als universitair docent bij de vakgroep Visteelt en Visserij. In september 1990 gaat hij naar Japan om aldaar post-doctoraal onderzoek te doen binnen het kader van een JSPS/NWO beurs.