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Androgenesis, gynogenesis and the production of clones in fishes: A review

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Abstract

Fish species that have external fertilization can be reproduced by induced parthenogenesis. The nuclear content of either the sperm or egg is destroyed by UV or gamma irradiation, and the treated gamete then is fused with an untreated egg or sperm to form a haploid embryo. This is subsequently made diploid by inhibition of either the second meiotic division or the first cell division. After first cell division blockage, the resulting individual is a so-called doubled haploid (DH). DH individuals carry only the duplicated set of chromosomes inherited from the untreated egg or sperm and are, by definition, fully homozygous.

In the first part of this review, we discuss the latest insights into the mechanisms underlying the process of making meiotic diploids and DH individuals, and review the problems associated with making and characteristics of doubled haploids and clones in fishes. In the second part of this review, we explore the use of doubled haploids and clones in quantitative trait locus mapping and selective breeding.

Keywords: Gynogenesis; Androgenesis; Doubled haploid; Clones; Zebrafish; Recombination; QTL; Fish; Teleost

Contents

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1. Introduction

Gynogenesis and androgenesis are terms that describe uniparental or parthenogenetic reproduction. In vertebrates, spontaneous gynogenetic reproduction occurs in reptilians, amphibians and teleosts. Spontaneous androgenesis, however, has never been observed. Reptilians reproduce by true parthenogenesis, i.e., without fertilization (thelytoky). All amphibian and teleost parthenogenetic species, on the other hand, require the stimulus of a sperm to initiate embryogenesis. Well-studied examples of natural gynogenetic fish species include the Amazon molly, *Poecilia formosa* (Schartl et al., 1995b), and the Gibel or Ginbuna, *Carassius auratus gibelio* (Cherfas, 1981; Yamashita et al., 1993). These species produce diploid (or sometimes triploid) oocytes which are activated by either homologous sperm or sperm from a closely related species. The mechanism by which diploid oocytes are produced varies. The most common form involves genome duplication, followed by normal cell division in which sister chromosomes preferentially pair. There is no recombination and segregation is avoided. The inheritance is thus strictly matroclinous. Nevertheless, in the Amazon molly, incorporation of small fragments of DNA (microchromosomes) from a bisexual host species has been reported (Schartl et al., 1995a). This is thought to counteract the deleterious effects of the accumulation of mutations that is likely to take place in this species. Diploid oocytes still require activation to initiate embryogenesis. In an elegant study with Ginbuna, Yamashita et al. (1990) demonstrated that fertilization with a functional sperm is necessary to initiate embryogenesis, but that the oocytes lack the necessary histokinases to break down the sperm nuclear envelope. The formation of a male pronucleus is thus effectively inhibited (Yamashita et al., 1990).

The fact that gynogenetic fish are viable has been taken as evidence for the absence of differential parental imprinting: male- and female-specific epigenetic methylation of certain genes during gametogenesis (Corley-Smith et al., 1996). Imprinting leads to unequal expression of maternal and paternal alleles in the embryo. Imprinting is common in mammals, where parthenogenetic animals are only viable when imprinting is prevented (Kono et al., 2004). Imprinting has not been observed in any lower vertebrate, including teleosts, but (de)methylation of DNA during early development is a widespread phenomenon (Altschmied et al., 2000).

Early attempts to produce gynogenetic fish in the laboratory focused on the production of diploids by suppression of meiotic divisions (i.e., meiotic diploids). In fishes, the second meiotic division is only completed shortly after ovulation and fertilization, and it was quickly discovered that this process could be effectively inhibited by cold-shocking the eggs (Makino and Ozima, 1943; Purdom, 1969; Cherfas, 1975; Nagy et al., 1978). However, the key publication which triggered widespread research in the production of homozygous gynogenetic (and later androgenetic) fish (i.e., mitotic diploids or doubled haploids) in the laboratory, came from George Streisinger (Streisinger et al., 1981). In this publication, a detailed description was given on how to produce meiotic diploids, mitotic

diploids and homozygous and heterozygous clonal lines of zebrafish (*Brachydanio rerio*). The paper also outlined the genetic consequences of the different treatments and the potential applications for research. Twenty-five years after that publication seems an appropriate point to examine the results which have followed from Streisinger's groundbreaking research. The publication marked the rise of zebrafish as the animal model for research on the embryonic development of vertebrates, but over the years, doubled haploids and clones have been abandoned as tools for research by the zebrafish community. To date, there are only a few fish species for which clones exist. In the present review we outline the progress that has been made since Streisinger's publication and discuss the problems that exist in making doubled haploids and clones. We start with a brief description of technical procedures and the efficacy of diploidization treatments to produce doubled haploids. Next, we discuss the performance of androgenetic and gynogenetic diploids, clones and F_1 hybrids, and review the use of these in quantitative trait locus (QTL) mapping and selective breeding.

In the literature, different names are used for parthenogenetic individuals, such as "gynogens" ("mitogynogens" and "meiogynogens") and "androgens" or "androgenotes" (see reviews by Pandian and Koteeswaran, 1998; Arai, 2001). In the present review we use the terms androgenesis and gynogenesis only to describe the methods to produce parthenogenetic animals. The animals themselves are termed meiotic diploids when they are produced by inhibition of the second meiotic division, and doubled haploids (DH), as in plants (Picard et al., 1994), when they are produced by inhibiting the first mitotic division (see Fig. 1). Clones are defined as groups of genetically identical fish. By crossing DH animals or animals from different clones, F_1 hybrids are created, which are groups of genetically identical (clonal), heterozygous animals.

2. Doubled haploids (DH)

DH animals have been produced in both marine and fresh water fish species, but most notably in the cyprinid and salmonid families. Table 1 summarizes publications on the production of DH fish by gynogenesis (G) and androgenesis (A). The process involves two steps. First, the DNA of either the sperm (in gynogenesis) or egg (in androgenesis) is fragmented by gamma (γ) or UV-irradiation. The treated gamete then is fused with an untreated egg or sperm to form a haploid embryo. Next, the haploid embryo is made diploid by inhibition of the first cell division (gynogenesis or androgenesis). This

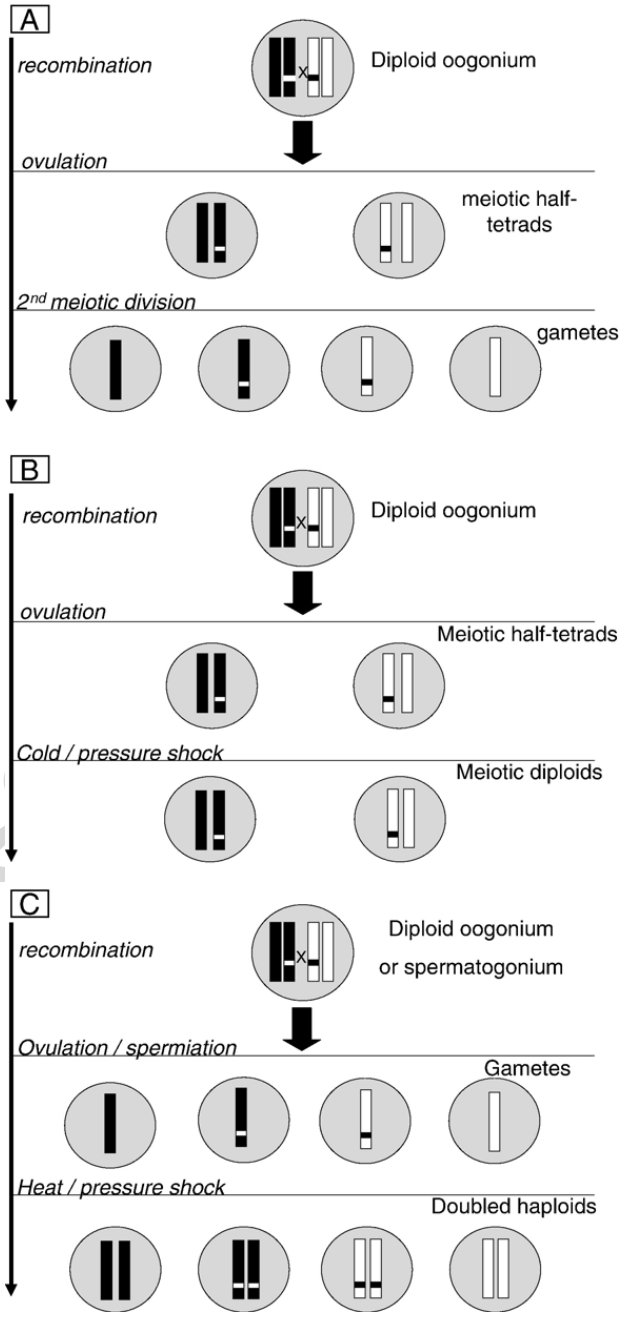

Fig. 1. Meiosis in fish (A) and consequences of restoration of diploidy by inhibition of the second meiotic (B) or first mitotic division (C). Legend: (a) Normal meiosis: recombination between non-sister chromatids, followed by the first meiotic division, produces two recombined sister-chromatid pairs. These are separated during the second meiotic division to produce four different recombined gametes. (b) Meiotic diploids are produced by inhibition of the second meiotic division. They are homozygous except for regions where crossing over took place. (c) Doubled haploids are produced by inhibition of the first mitotic division of the haploid embryo. They are homozygous diploid.

typically is done by a heat or pressure shock administered around the time of the prophase of the first mitotic division. The optimal times for applying heat as compared to pressure shocks appear to be slightly

Table 1
Summary of fish species for which gynogenetic (G) or androgenetic (A) doubled haploids have been produced

Common name	Species name	Type	Irradiation	Genome duplication	Yield (%)	Reference
Cyprinidae						
Zebrafish	*Brachydanio rerio*	G	UV	CEP/*T* (41/2)	11	Streisinger et al. (1981)
		G	UV	CEP	9	Hörstgen-Schwark (1993)
		A	X-ray	*T* (41.5/2)	2.1	Corley-Smith et al. (1996)
Medaka	*Oryzias latipes*	G	UV	*T* (41/3)	5	Naruse et al. (1985)
				P (700)	2	
Common carp	*Cyprinus carpio*	G	UV	*T* (40/2)	15.5	Komen et al. (1991)
		G	γ	*T* (40/1.5)	10.6	Yousefian et al. (1996)
		A	X-ray	*T* (40.5/2–3)	9	Grunina et al. (1990)
		A	UV	*T* (40/2)	19.8	Bongers et al. (1994)
Ornamental carp	*Cyprinus carpio*	G	UV	*T* (40/2–3)	8	Cherfas et al. (1994)
Grass carp	*Ctenopharyngodon idellus*	G	UV	*T* (41/2)	<1	Li and Luo (2003)
Goldfish	*Carassius auratus*	G	UV	*T* (40/1)		Nagoya et al. (1990)
		A	γ	*T* (40/2)	28	Bercsenyi et al. (1998)
Crucian carp	*Carassius auratus gibelio*	A	X-ray [1)]	*T* (420/2–3)	<0.1	Grunina and Neifakh (1991)
Mud loach	*Misgurnus mizolepis*[2)]	A	UV	*T* (40/2)	19	Nam et al. (2002)
		G	UV	*T* (40.5/2>0/45)	35	Nam et al. (2004)
Loach	*Misgurnus fossilis*	G		*P* (800)		Suwa et al. (1994)
Rosy bitterling	*Rhodeus ocellatus ocellatus*	G	UV	*T* (0,60)	17.8	Kawamura (1998)
Rosy barb	*Puntius conchonius*	A	UV	*T*	14 (7[3])	Kirankumar and Pandian (2004)
Tiger barb	*Puntius tetrazona*	A	UV	*T* (41/2)	15	Santhakumar et al. (2003)
Salmonidae						
Ayu	*Plecoglossus altivelis*	G	UV	*P* (650)		Han et al. (1991)
Rainbow trout	*Oncorhynchus mykiss*	G	γ	*P* (530)	<1	Chourrout (1984)
		G	UV	*P*		Foisil and Chourrout (1992)
		G	UV	*T* (30/9)	23	Diter et al. (1993)
	Inbred sperm	A	γ	*P* (680)	4.8	Scheerer et al. (1986)
	Outbred sperm				6.2	
	Tetraploid sperm	A	γ	No	11.8	Thorgaard et al. (1990)
Amago salmon	*Oncorhynchus masou ishikawae*	G	UV*)	*P* (650)	5.6	Kobayashi et al. (1994)
		A	γ	*P* (650)		Nagoya et al. (1996)
Other fresh-water species						
Siberian sturgeon	*Acipenser baerii*	A	X-ray	*T* (37/30)	12.2	Grunina and Neifakh (1991)
African catfish	*Clarias gariepinus*	G	UV	*T* (40/1)	<1	Galbusera et al. (2000)
Nile tilapia	*Oreochromis niloticus*	G	UV	*P* (630)/*T*(41/3.5)	1–2	Hussain et al. (1993)
		G	UV	*T* (41/6)	0.85	Müller-Belecke and Hörstgen-Schwark (1995)
		A	UV	*T* (42.5/3–4)	5.8[4)]	Myers et al. (1995)
		G	UV	*T* (42.5/3)	4.3	Sarder et al. (1999)
		A	UV	*T* (42.5/3–4)	0.07	Karayucel et al. (2002)
Hybrid tilapia	*O. niloticus*×*O. aureus*	A	UV	*T* (41.6/5)	0.9	Marengoni and Onoue (1998)
Muskellunge	*Esox masquinongy*	G	UV	*T* (31/9)	16	Lin and Dabrowski (1998)
Fighting fish	*Betta splendens*	G	UV[5)]	*P* (530)	21	Kavumpurath and Pandian (1994)
Marine species						
Red sea bream	*Pagrus major*	G	UV[6)]	*P* (700)	–	Kato et al. (2001)
Hirame	*Paralichthys olivaceus*	G				(Tabata and Gorie, 1988; Tabata, 1997)
European sea bass	*Dicentrarchus labrax*	G	UV	*P* (830/930)	8	Francescon et al. (2004)

1) common carp eggs as donor. 2) transgenic sperm. 3) cryopreserved sperm from *Puntius conchonius*. 4) yield to yolksac larvae. 5) tilapia sperm. 6) frozen sperm from *P. gonionotus.* *) rainbow trout sperm.
G=gynogenesis; A=androgenesis; Yields are % hatched fry; *T*=temperature and duration for heat shock (°C/min), *P*=pressure shock (in kg/cm^2), CEP=combined ether/pressure shock.

different (Palti et al., 1997, 2002). A brief description of these techniques is given below.

2.1. Genome inactivation by DNA irradiation

DNA can be destroyed by ionizing irradiation, such as 60Co gamma (γ) irradiation or X-rays. Both X-rays and γ-irradiation penetrate deep into cell tissue and effectively fragment the DNA. For these reasons, ionizing radiation has been considered the method of choice for irradiating larger volumes of sperm or large fish eggs, such as those of salmonids (Arai et al., 1979). Ionizing radiation has the disadvantage that few laboratories are allowed to have radioactive sources outside special containment areas. Most salmonid eggs can be stored for up to 24 h before fertilization, and thus can be transported to a source of γ-irradiation. However, for most teleost eggs, post-ovulatory viability is limited to only a few hours and this requires that irradiation be performed as close as possible to the site where gametes are collected and androgenesis or gynogenesis is performed.

Storage conditions for sperm are generally less critical than those for eggs, provided that an appropriate extender and storage temperature be used. Because γ-irradiation is homogenous, few sperm should escape DNA destruction; the critical minimum dose required to avoid paternal DNA contamination would be $>1.1\times10^5$ R (Thorgaard et al., 1985). At lower doses, there is a substantial risk that small paternal DNA fragments persist and will be expressed in gynogenetic progeny (Chourrout and Quillet, 1982; Disney et al., 1987).

In contrast to γ-irradiation, UV-irradiation is cheap and simple to set up. In most cases, germicidal bulbs emitting UV light at 254 nm are used. DNA inactivation is optimized by varying the distance between the lamp and the sample, and thus the intensity, and by varying the duration of irradiation. UV-254 nm has very low penetration power and only small, shallow samples of sperm or eggs can be irradiated (Palti et al., 1997). Larger volumes (up to 10 ml) can be irradiated provided that the sample is stirred and refrigerated during irradiation (Komen et al., 1988). Trials to use UV on large fish eggs (e.g., those of salmonids) thus far have been unsuccessful. However, smaller eggs (diameter<2 mm) can be irradiated successfully. Cyprinid eggs need to be constantly stirred in a synthetic ovarian fluid to prevent precocious activation (Bongers et al., 1994). Unfertilized tilapia eggs are not activated in water and thus synthetic ovarian fluids can be avoided (Myers et al., 1995).

The primary effect of UV on DNA is dimerization of adjacent pyrimidines (Friedberg, 1985), causing DNA–DNA and DNA–protein cross-linking. Other changes are the formation of hydrates of cytosines, and base-pair substitutions (Cieminis et al., 1987). The ultimate result is chromosome fragmentation, similar to γ-irradiation. In plants, this feature can be used to produce asymmetric somatic hybrids (Forsberg et al., 1998). Sub-optimal UV-irradiation of eggs or sperm also can produce chromosome fragments which persist in gynogenetic or androgenetic progeny (Arai et al., 1992; Quillet, 1994; Lin and Dabrowski, 1998; Bertotto et al., 2005). However, if properly done, the yields of gynogenetic fry obtained with UV-irradiated sperm are higher than those obtained with γ-irradiated sperm (Foisil and Chourrout, 1992). For this reason and because of the problems relating to access to ionizing radiation sources, UV has become the favored method for irradiating sperm.

2.2. Genome duplication

Genome duplication is achieved by inhibiting the first mitotic division through pressure or heat shock. If applied around the prophase of mitosis, this results in duplication of the haploid chromosome set, followed by normal cell divisions.

Pressure shocks can be administered with a custom-made or commercially available press. The pressure applied to induce genome duplication typically varies from 530 to 800 kg/cm^2 (52–78 MPa or 7538–11,378 psi). Heat shocks are very simple to apply. A water bath with good thermoregulation is the only requirement. Heat shocks are particularly suited for benthic eggs or eggs that stick to a substrate, such as carp eggs. Such eggs can be attached to a mesh substrate which is lifted from one water bath to the other. Optimal temperatures for heat shocks are species-specific and vary from 27 to 36 °C for salmonids to up to 42 °C for tropical fishes, such as tilapia. For most cyprinid species, 40–41 °C is considered optimal. Effective heat shock temperatures are close to the upper limit of tolerance as they act through depolymerization of protein complexes. Finding the right combination of temperature and duration is therefore critical. Pressure shocks are more difficult to standardize and to apply to large volumes of eggs than temperature shocks, which is why some researchers working with cleavage blockage in salmonids prefer to work with heat shocks.

The mechanism leading to genome duplication generally is assumed to be abortion of mitosis as a result of spindle depolymerization, followed by endomitosis. Microtubules are sensitive to heat and pressure shocks, as well as to chemical insult (Dustin, 1984). However, heat and pressure shocks also cause destabilization and disorganization of other organelles, including

centrosomes (Debec and Marcaillou, 1997; Vidair et al., 1993). Centrosomes are microtubule-organizing centers. Two centrosomes are needed to form the mitotic spindle; each centrosome contains two centrioles. Centrosomes duplicate during the S phase of the cell cycle by "budding" of the centrioles. This duplication is semi-conservative; each newly-formed centrosome contains an original centriole and its newly-formed daughter (Delattre and Gonczy, 2004). Recently, it was shown in amago salmon that, following a pressure or heat shock, microtubules are realigned and the first mitotic division proceeds normally. However, during the second mitotic division, two monopolar spindles are formed and this division subsequently is aborted (Zhang and Onozato, 2004). The explanation of this phenomenon could be that the daughter centrioles are more sensitive to physical insult than the mother centrioles. Heat and pressure shocks will destroy the daughter centrioles, but the two centrosomes (now each with one mother centriole) are still capable of rebuilding the spindle. However, in the next cell cycle, the centrosomes will fail to duplicate due to lack of daughter centrioles.

2.3. Yields of doubled haploids

Table 1 summarizes yields of hatched androgenetic and gynogenetic doubled haploids from a number of species. It should be noted that this table only summarizes successful attempts. For many species, production of viable gynogenetic and/or androgenetic doubled haploids has been tried but not achieved (e.g. androgenesis in muskellunge: Lin and Dabrowski, 1998).

In general, the yields of gynogenetic fry appear to be similar to those of androgenetic fry. A precise comparison is complicated by the fact that some authors report yields relative to control fertilizations while others use absolute yields. Different authors also use different end-points, e.g., eye-stage (as in salmonids), hatching or first-feeding.

In general, the reported yields of DH individuals range between 1 and 20%. In the original study with zebrafish, Streisinger et al. (1981) reported yields of gynogenetic DH as high as 20% following a combined ether/pressure shock. In a more recent study, yields of up to 30% were obtained using the same combined treatment (Hörstgen-Schwark, 1993). Yields of androgenetic diploid zebrafish, on the other hand, have been very low (Corley-Smith et al., 1996). In common carp and rainbow trout, the best studied species, (absolute) yields of first-feeding diploid DH fry can be as high as 19% but more typically vary between 5 and 10% (Parsons and Thorgaard, 1985; Komen et al., 1991; Bongers et al., 1994). For others, such as tilapia and some marine species, yields appear to be much lower (<5% of treated eggs).

2.3.1. Tetraploidy

It is not clear why there is such a big difference in yield between salmonids and cyprinids on the one hand, and many other species. One explanation is that some species will not tolerate diploidization of the haploid genome while other species will, because, as a species group, they share a recent evolutionary history of genome duplication. Teleost fishes have undergone multiple genome duplications, as exemplified by, e.g., the number of *Hox* clusters (Amores et al., 2004; Moghadam et al., 2005). Duplicated genes are subject during evolution to degenerative mutations, neo-functionalization and sub-functionalization (Force et al., 1999), leading to greater genetic diversity. However, in both the salmonid and cyprinid families, genome duplications have taken place in recent history which produced animals with 100 chromosomes or more (Allendorf and Thorgaard, 1984; Kirpičnikov, 1987; Phillips and Rab, 2001; David et al., 2003). These species still retain many genes as functional duplicates (Futami et al., 2001; Brunelli et al., 2001; Fredriksson et al., 2004). To tolerate genome duplication, gene dosage compensation mechanisms had to evolve simultaneously, and it is possible that such compensation mechanisms help to overcome the effects of haploid genome doubling. Rainbow trout tetraploids and many cyprinid polyploids are examples of species that successfully evolved dosage compensation mechanisms to overcome the effects of multiple gene sets (Postlethwait et al., 2004).

2.3.2. Genetic load

Doubled haploids produced by androgenesis or gynogenesis theoretically should suffer from inbreeding depression due to the expression of homozygous deleterious mutations. Many of these mutations could act during early embryo development, thereby causing a significant reduction in survival of DH fry. In wild *X. laevis*, obtained from indigenous African populations, gynogenesis and inbreeding were used to isolate mutations affecting development. A genetic load of 1.875 developmental mutants/female was found, which is only slightly less than the load of mutants with major developmental effects found in *Drosophila* and man (Krotoski et al., 1985).

In fishes, there are no estimates of genetic load in terms of early embryonic recessive deleterious mutations. However, assuming an average genetic load of 1–2 harmful recessive genes for any female parent, the mortality in her doubled haploid progeny could be as high as 50–75%. There is some conflicting evidence as to whether yields (first-feeding fry) are influenced by expression of homozygous deleterious mutations during embryonic development. In rainbow trout (Scheerer

et al., 1986, 1991), sperm from (partially) inbred and outbred sources gave similar yields of androgenetic fry. Similar results were reported by Babiak et al. (2002). A recent study showed similar yields of haploid androgenetic rainbow trout fry from homozygous clonal and outbred sources (Patton et al., 2007). However, others observed improved yields of meiotic diploid *O. aureus* tilapia after four successive rounds of gynogenesis. An analysis with microsatellite markers revealed a segregation distortion at three unlinked markers with deleterious effects between four and eight days after fertilization (Palti et al., 2002).

In common carp, hatched yields of androgenetic fry were very similar, regardless of whether homozygous inbred sperm or sperm from semi-wild carp was used (Bongers et al., 1994; Tanck et al., 2001a). In both these reports, the same (genetic) egg source was used. However, screening with 11 microsatellites revealed a significant segregation distortion for two microsatellite loci in androgenetic DH progeny from semi-wild carp (Tanck et al., 2001a). Together, these results suggest that the effects of genetic load may be present but when yields of DH embryos vary so widely as a result of treatment × egg quality interaction effects (e.g. from 1–10%), then the effects of deleterious mutants might easily go unnoticed.

2.3.3. Egg quality

Egg quality effects are one of the main reasons that have been put forward to explain low yields of doubled haploid fry. Almost every author cited in Table 1 has reported significant female effects on the sensitivity of the eggs to heat or pressure shocks. In gynogenesis, it is not possible to disentangle egg quality effects from genetic load effects. However, using androgenesis, these effects can be separated. In rainbow trout, Babiak et al. (2002) observed that outbred females gave significantly higher yields of androgenetic DH than did inbred females. Significant differences in yield of androgenetic haploid rainbow trout fry were also observed among outbred females (Patton et al., 2007). In common carp, Bongers et al. (1995) observed significant differences when different F_1 hybrid egg sources were fertilized with genetically identical sperm sources (see also Table 2). These F_1 hybrids were obtained by crossing two homozygous F_1 parents and thus were free of any recessive maternal mutations that could affect embryonic development. In zebrafish, gynogenesis was used to identify 14 recessive maternal effect mutations. Homozygosity for these mutations in adult females leads to the inviability of their offspring (Pelegri et al., 2004).

Egg quality is a complex phenomenon and still poorly understood (reviewed by Kjørsvik et al., 1990). Some factors that improve fertilization and survival of normal fry, such as fatty acid composition in marine species, might also play a role in determining survival after heat or pressure shocks. Other factors more directly relate to timing of stripping in relation to maturation and ovulation. With hormonally induced maturation and ovulation, stripping is normally attempted after a predetermined number of hours following the last injection (latency time: defined by dose and temperature). For those species where ovulation cannot be reliably induced by hormonal treatment (e.g., rainbow trout, tilapia and zebrafish), the time of ovulation and stripping is inferred from mating

Table 2
Summary of species for which homozygous clones and/or F_1 hybrids have been produced

Common name	Species name	G/A*)	Clone	F_1 hybrid	Reference
Zebrafish	*Brachydanio rerio*	G	+	+	Streisinger et al. (1981)
Medaka	*Oryzias latipes*	G	+	+	Naruse et al. (1985)
Common carp	*Cyprinus carpio*	G	+	+	Komen et al. (1991)
		A	+	+	Bongers et al. (1997c)
		G	+	+	Ben-Dom et al. (2001)
Nile tilapia	*Oreochromis niloticus*	G	+	+	Müller-Belecke and Hörstgen-Schwark (1995)
		G	+	+	Hussain et al. (1993)
		A	?	+	Sarder et al. (1999)
Amago salmon	*Oncorhynchus rhodorus*	G	+	+	Kobayashi et al. (1994)
		A	+		Nagoya et al. (1996)
Ayu	*Plecoglossus altivelis*	G	+	+	Han et al. (1991)
			+	+	Takagi et al. (1995)
Rainbow trout	*Oncorhynchus mykiss*	G	?	+	Quillet (1994)
		A	+		Scheerer et al. (1991)
		A	+	+	Young et al. (1995)
Hirame	*Paralichthys olivaceus*	G	+	+	Hara et al. (1993)
Red seabream	*Pagrus major*	G	+	+	Kato et al. (2002)

*) G = gynogenesis, producing female clones; A = androgenesis, producing male clones.

behavior. In both cases, the correct timing of stripping is important. Precocious stripping could bring out immature eggs. Aging and over-ripening results in destabilization of the meiotic spindle configuration, which could produce more aneuploid embryos, as has been observed in hamsters (Hansmann et al., 1989). In common carp, over-ripe eggs tend to give poorer results in androgenesis and gynogenesis. In trout, it is not uncommon to check females only 1–2 times a week for signs of ovulation. This could lead to large variation in egg age and might explain why trout egg sources sometimes show large variations in DH production.

2.3.4. Asynchronous embryo development

Attempts to induce mitotic gynogenesis often result in the production of a small percentage of heterozygous animals among the presumed DH fry. Genetically, they resemble half-tetrads: the diploid chromosome set consists of maternal sister chromatid pairs (Streisinger et al., 1981) and apparently result from failure to extrude the second polar body (Fig. 1). Many authors have reported on the occurrence of such spontaneous diploids in DH progenies (Komen et al., 1991; Cherfas et al., 1994). In African catfish the majority of presumed DH fry produced over a range of 40 min after fertilization, turned out to be meiotic diploids (Galbusera et al., 2000). Similar observations were made in rainbow trout (Young et al., 1996) and European sea bass, *Dicentrarchus labrax* (Francescon et al., 2004). The origin of these meiotic diploids is not clear. Sometimes a genetic predisposition of the female parent to produce (large numbers of) spontaneous meiotic diploids is observed (Cherfas et al., 1995; Ezaz et al., 2004c). Another explanation is that they originate from "mechanically stripped" immature eggs that still need to complete meiosis (see Fig. 1). However, they also might result from "late fertilization". In marine fishes, sperm and eggs retain their fertilizing capabilities far longer than for freshwater fish, where fertilization for many species needs to be almost instantaneous. Both explanations emphasize the importance of uniformity in induced ovulation and fertilization procedures.

Several authors have pointed out that the timing of the temperature or pressure shock should coincide with the prophase of the first mitotic division (Nagoya et al., 1990; Komen et al., 1991). However, embryonic development is probably highly asynchronous, as evidenced by the fact that the optimal "window" for H/P shocks can be several minutes. In common carp and zebrafish, a comparison has been made between window length and yields obtained when embryos were shocked immediately after fertilization (when all eggs are in prophase of the second meiotic division) and those of embryos shocked to inhibit the first mitosis. In the first case, the window was only 1–2 min and the yields could be as high as 50%. In the second case, the optimal window was 6–8 min, and the yield was correspondingly lower (Komen et al., 1991; Hörstgen-Schwark, 1993). However, there is no reduction in the length of the optimum window when comparing genetically uniform F_1 hybrids with outbred female common carp.

It has been noted in zebrafish that the use of ether in combination with H/P shocks can increase the yields of DH fry. Ether destabilizes microtubules and one of the effects could be that it "captures" embryos as they enter mitosis, thereby causing synchronisation (Streisinger et al., 1981; Hörstgen-Schwark, 1993). More recently, it was shown that yields of mud loach could be improved significantly when a heat shock of 40 °C was followed by a cold shock (0 °C) for 45 min (Nam et al., 2004). The success of such combined treatments is difficult to explain from synchronization alone, and more likely relates to the combined effect of physical shocks on centrosomes and the mitotic spindle (Debec et al., 1990; Rousselet et al., 2001).

2.4. Survival of doubled haploids

Doubled haploids can suffer considerable mortality in the first weeks or months after first-feeding. Streisinger et al. (1981) reported survival rates of up to 30% for mature 3-month-old homozygous zebrafish. Hörstgen-Schwark (1993) observed survival rates of 4–20% of DH zebrafish, produced by a combined pressure/ether shock. The survival of DH produced by heat shocks was much lower. The survival rate of androgenetic DH zebrafish has not been measured, although Corley-Smith et al. (1996) mention the survival of one putative androgenetic male to adulthood. Naruse et al. (1985) obtained 10 hatched fry of medaka, of which four survived to adulthood. In the rosy bitterling, survival of DH was 5.5% between hatching and 30 days post-hatch (Kawamura, 1998). In tiger barb, 7% of the androgenetic DH survived up to maturity (Santhakumar et al., 2003). Kobayashi et al. (1994) mention that only six out of 98 first-feeding DH amago salmon survived until the spawning season two years later.

In other species, mortality is less dramatic. In tilapia, 47% of putative gynogenetic DH survived until day 60 (Müller-Belecke and Hörstgen-Schwark, 1995). Among the DH trout surviving to first-feeding, survival to 12 months is in general between 40 and 70% (Scheerer et al., 1986), but much lower survival rates of 2% (Quillet, 1994) or even less (<0.8%; Babiak et al., 2002) have been reported as well. In the European seabass, 48% of doubled haploids from one particular family

survived until 284 days (Francescon et al., 2004). In the common carp, survival between first-feeding and 10 weeks typically varies between 50 and 80% (Table 2). Of these, almost all subsequently survive to maturity. There is no difference in survival rates between gynogenetic and androgenetic DH (Komen et al., 1991; Tanck et al., 2001a).

In many cases mortality is directly linked to severe deformation, especially in the cranium and vertebral column. Such deformations interfere with feed uptake and can result in death from starvation or diseases (Müller-Belecke and Hörstgen-Schwark, 1995). Several reasons have been put forward as causes of these deformities, such as the effects of gamma or UV-irradiation on mitochondria and other maternal components, the effects of the heat/pressure shock, and inbreeding depression. These will be discussed below.

2.4.1. Maternal damage

Both γ- and UV-irradiation can have severe effects on development of oocytes and early embryos (Egami and Ijiri, 1979). Theoretically, γ-irradiation can produce point mutations or deletions within the mitochondrial DNA or cytoplasmatic mRNA (Thorgaard et al., 1990). It is also known that UV-irradiation can cause pyrimidine-dimer formation and DNA–DNA or RNA–RNA cross-linking (Friedberg, 1985). Only a few studies have addressed the question whether mitochondrial DNA is damaged during irradiation. In these studies, no evidence for mitochondrial damage has been found, using either RFLP analysis (May and Grewe, 1993) or full-length mitochondrial DNA sequencing (Brown and Thorgaard, 2002). Similarly, there has been no evidence for mitochondrial damage caused by UV (Myers et al., 1995). It is possible that the mitochondrial DNA is protected from the harmful effects of γ-irradiation by its double membrane and small circular genome (May and Grewe, 1993). In most fish eggs, mitochondria are concentrated in the Balbiani body, which is embedded in the yolk. This large amount of yolk will certainly protect the mitochondria from UV-irradiation. In addition, the large copy number of mitochondria within the fish oocyte may mask any minor loss or damage.

The effects of radiation on other maternal components such as mRNA have been less studied. It has been suggested that egg irradiation does not have a great effect on embryo survival (Parsons and Thorgaard, 1985; Thorgaard et al., 1990; Myers et al., 1995). In a series of experiments with common carp, using identical F_1 hybrid egg and sperm genotypes, it was shown that the amount of fluctuating asymmetry and deformity in the cranium was somewhat higher in androgenetic DH carp produced from UV-irradiated oocytes than in gynogenetic DH carp produced with UV-irradiated sperm (Bongers et al., 1997c). However, there were also considerable differences between (genetically identical) androgenetic DH groups. The cause of these differences is not clear and could be random (i.e. technical/experimental) variation or the result of specific maternal–paternal incompatibility. In trout, there is evidence for specific male–female interactions on embryo viability (Patton et al., 2007).

2.4.2. Effects of heat/pressure shocks

As stated earlier, heat and pressure shocks cause destabilization and disorganization of microtubules and centrosomes. Heat-shocked Chinese hamster ovary (CHO) cells show heat-induced alterations to the centrosomes which result in multipolar mitotic spindles, delay in prophase–metaphase, and formation of multinucleated cells (Vidair et al., 1993; Debec and Marcaillou, 1997). All of these effects result in cell death. In amago salmon, Zhang and Onozato (2004) showed that in some pressure-shocked embryos, centriole irregularities were still observed in blastomeres undergoing the third mitotic division, resulting in mosaic haploid/diploid individuals. Similar results were obtained by Suwa et al. (1994), and by Hussain et al. (1993), who noted numerous aneuploid and near-diploid embryos among presumed gynogenetic DH progeny. These results show that the effects of heat and pressure shocks on fish embryos can be comparable to those observed in *Drosophila* or in CHO cells, and may constitute an important cause of embryo deformity.

Heat and pressure shocks not only affect mitosis, but probably also a whole range of other mechanisms related to early embryo development. In goldfish, it was shown that heat shocks induced several developmental disorders such as thin blastodisc formation, delay of epiboly, and deficiency of dorso-anterior structures. Less, and sometimes reduced, signals of *goosecoid* mRNA, a dorsal mesodermal marker, were observed in embryos treated with heat-shock at 40 °C for 1 min at 5 min post-fertilization. These results suggest that such treatments affect not only cell division, but also influence dorso-ventral differentiation (Yamaha et al., 2002).

2.4.3. Inbreeding depression

In common carp, six wild males were crossed with the same homozygous female to produce six paternal half-sib families, heterozygous for any male-derived recessively expressed mutations (Tanck et al., 2001a). From each half-sib family, 5–6 sires were propagated by androgenesis to produce 36 full-sib families of DH fry. Yields of first-feeding DH fry were typically 10–15%, not different

from androgenesis experiments with homozygous (DH) sperm sources. However, during subsequent rearing, large differences became apparent. The average survival over all six paternal half-sib groups was 47±22.5%. In two half-sib groups, the survival was 60±22% and 65±16%, respectively. In a third half-sib group, 21.8±14% of DH fry survived; almost 50% were lost within one week, apparently due to a metabolic disorder. These survival rates were comparable to those obtained with gynogenetic DH fry (Komen et al., 1991) and were significantly lower than what is normally observed when homozygous inbred sperm is used. One popular explanation is that these low and variable survival rates are caused by expression of deleterious genes and inbreeding depression. However, in rainbow trout, there is little evidence that inbreeding depression influences survival rates of doubled haploids (Scheerer et al., 1986, 1991; Babiak et al., 2002) or haploids (Patton et al., 2007).

2.5. Sex ratios in doubled haploids

Gynogenesis and androgenesis can be used to obtain information on the sex-determining mechanism in fishes. In species with XX–XY sex determination, gynogenetic progeny are expected to be all female. Androgenetic progeny should segregate as XX and YY animals, assuming that YY individuals are viable, and YY males should sire all male progeny. These expectations are reversed with species in which males have ZZ and females ZW sex chromosomes (reviewed in Devlin and Nagahama, 2002). In practice however, sex ratios can vary from all female to all male gynogenetic doubled haploids. Some of these results can be attributed to environmental factors overriding the sex-determining system. In other cases, there are clear indications that recessive mutations affecting sex determination are segregating.

Gynogenetic DH zebrafish are predominantly male, and even DH females can produce all-male clones when reproduced by gynogenesis (Streisinger et al., 1981; Müller-Belecke and Hörstgen-Schwark, 1995). These aberrant sex ratios appear to be a consequence of the fact that in zebrafish, all animals initially develop as females, after which a variable proportion will change sex into male (Maack and Segner, 2003).

In Nile tilapia (*O. niloticus*) originating from Lake Manzala, gynogenetic DH males have been observed on several occasions (Sarder et al., 1999). Sex determination in Nile tilapia is thought to be genetic and XX–XY (Carrasco et al., 1999), but can be overruled by temperature (Devlin and Nagahama, 2002). Müller-Belecke and Hörstgen-Schwark (1995) observed a high proportion of males (35.3%) among gynogenetic doubled haploids. Gynogenetic reproduction of six DH females led to all-female homozygous clones, but progenies of five DH males mated with different females were either mostly female (four) or predominantly male (one). The authors assumed that 1–2 minor sex-determining factors, which are able to override the XX–XY mechanism when they occur in the homozygous state, might account for the sex ratios observed. Sarder et al. (1999) observed varying numbers of males in gynogenetic progeny groups (average 20% males). They concluded that more than one minor sex-determining locus was segregating in their population. More recently, Karayucel et al. (2004) confirmed the existence of one of these recessive genes causing female-to-male sex reversal, as it appears to be linked to red color in this *O. niloticus* population.

Ezaz et al. (2004a) also examined sex determination and departures from predicted sex ratios in DH Nile tilapia. YY males were produced by androgenesis from XY male parents and by mitotic gynogenesis from XY neofemale parents. Progeny testing of androgenetic and gynogenetic males and gynogenetic females, in general confirmed the predictions based on presumed sex chromosome constitution. Androgenetic and gynogenetic males sired all-male progeny. Gynogenetic females sired all female groups. However, there were significant differences among androgenetic families with respect to deviations from expected progeny sex ratios. Among gynogenetic families, the largest deviations occurred in the same family for both male and female gynogenetic DHs. The authors concluded that "the factors that cause departures from the sex ratios predicted by chromosomal sex determination appear to be autosomal, heritable, polymorphic and able to influence sex ratios in both directions" (Ezaz et al., 2004a).

Rainbow trout (*Oncorhynchus mykiss*) also have an XX–XY type of sex determination. Gynogenetic DH progeny are usually all female. Androgenetic DH males sire all-male progeny which supports the hypothesis that these are YY (Scheerer et al., 1991; Nagoya et al., 1996). However, Quillet et al. (2002) recently reported the genetic analysis of sex reversal in a gynogenetic DH family of rainbow trout consisting of 13 males and 14 females. The transmission of maleness was studied over three generations, using both conventional and/or meiotic diploid and DH offspring. On the whole, males as well as intersexes were observed in crosses between an expected carrier male and carrier female, and in gynogenetic offspring of expected carrier females, but not in crosses between expected carriers and wild-type control animals. Sex ratios in the different crosses in most cases fit Mendelian proportions based on a model

that assumes the existence of a recessive mutation in a sex-determining gene. Instances of apparent sex reversal have been observed in doubled haploid rainbow trout produced by androgenesis, although it is unclear whether these have genetic or environmental causes (Felip et al., 2004).

There is a striking similarity between these observations on sex ratios in tilapia and trout, and observations on sex ratios in DH progeny of common carp. In carp, sex determination is XX–XY, based on crosses with XX-sex reversed or androgenetic YY males (Bongers et al., 1999). Several gynogenetic DH progenies have been produced with different genetic backgrounds; (Komen et al., 1991; Bongers et al., 1997b). Most progenies were all-female, but equal numbers of females and males (and intersexes) were detected in one DH progeny group from an F_1 parent of a cross between two different strains ("Domesticated" and "Wild", described in Komen et al., 1992a). A subsequent genetic analysis indicated that the sex reversal could be explained by assuming the segregation of a recessive mutation, termed *mas-1* (Komen et al., 1992a). In homozygous condition, this mutation overrides normal female sex differentiation and causes (partial) sex reversal of ovaries into (ovo)testes (Komen et al., 1992b). Recently, it was discovered that both homozygous clones of XX males, which are presumed carriers of the *mas-1* mutation, also are suffering from adrenal hyperplasia, a condition that in humans causes masculinization of female external genitalia (Ruane et al., 2005).

Female-to-male sex reversal also has been observed in gynogenetic DH of marine species, such as the hirame or Japanese flounder (*Paralichthys olivaceus*) (Yamamoto, 1999) and the red sea bream, *Pagrus major* (Kato et al., 2001, 2002). Given its occurrence over such a wide range of species, it has been postulated that sex reversal in DH progeny might be the result of increased developmental instability caused by the homozygous condition (Scheerer et al., 1991). In all cases where sex reversal has been subjected to genetic analysis, recessive factors have been identified that appear to interfere with normal female gonadal differentiation. Sex differentiation in fishes is unstable as compared to mammals. Sex can be changed by environmental factors, such as temperature, and by changes in plasma sex steroid profiles (reviewed in Devlin and Nagahama, 2002). This means that any regulatory or structural imbalance in the cascade of gene expression during sex differentiation could result in sex reversal. In heterozygous animals these imbalances are buffered, but in homozygous condition they may become expressed. From this point of view, gynogenesis and androgenesis should be excellent genetic tools to unravel this complex cascade of gene expression.

2.6. Fertility of doubled haploids

In general, the effects of inbreeding are first noticed in fertility-related traits, especially in females (Keller and Waller, 2002). In fishes, female reproduction is an extremely complicated process involving a number of critical checkpoints such as meiosis, maternal RNA production and expression, yolk formation, ovulation and fertilization. Fertility problems in females are generally related to delayed natural spawning time, decreased ovulation response to hormonal induction and reduced egg size and quality (Kjørsvik et al., 1990).

Only a few detailed studies have been performed on the fertility of DH progeny. In common carp, 30–35% of the gynogenetic DH females produced fertile eggs after hormonal stimulation. Variation in gonado-somatic index (GSI) and egg size were increased and egg quality was reduced. In androgenetic progeny, sterility can vary from 13 to 94%, and fertility in females is even more reduced. Of the 48 presumed females produced by androgenesis, only 4 produced viable eggs (Bongers et al., 1999). In androgenetic tiger barbs (*Puntius tetrazona*) on the other hand, performance of DH females in terms of fecundity and fertility of eggs was only little less than that of outbred females (Santhakumar et al., 2003). It is not clear why DH tiger barbs are so different from other DH species.

In tilapia, 10 of 77 gynogenetic DH females produced viable eggs. Five of these females came from the same dam (Müller-Belecke and Hörstgen-Schwark, 1995). In rainbow trout, fertility of androgenetic DH females is also severely reduced (Scheerer et al., 1991). Quillet (1994) reported that the proportion of gynogenetic DH females that spawned for the first time at age two years was smaller than for non-inbred females. Absolute fecundity of DH females and survival of embryos from eggs of DH females also was reduced (29 and 50% of control values at 2 and 3 years, respectively). The poor fertility effectively inhibited the production of homozygous clones. The same problem was reported by Arai (2001) who was not able to reproduce gynogenetic DH loach to produce homozygous clones. Marine fish are not very different in this respect. For red seabream, only one of 13 DH females could be successfully reproduced (Kato et al., 2002).

Interestingly, fertility in DH males (YY) is hardly affected at all, at least not in rainbow trout and common carp. In some other species, GSI and sperm counts of YY males were higher than those of XY males (e.g. tiger barb: Santhakumar et al., 2003). Androgenetic YY males of Nile tilapia are also fertile and sire all-male progeny (Sarder et al., 1999), but outbred YY males (obtained by

crossing hormonally sex-reversed parents) have lower testosterone levels than XY males (Rowell et al., 2002).

The fertility of DH males suggests that testis development and sperm production is less sensitive to genetic load and inbreeding depression than is oocyte development. There could be several reasons for this. Work on triploid fishes has shown that females are sterile, but that males can still produce sperm, even when this sperm is aneuploid due to aberrant meiosis. Sperm also is not sensitive to the presence of chromosomal fragments. Such fragments significantly impair fertility in females (Krisfalusi et al., 2000). Second, sperm are far less complex cells than oocytes, consisting basically of a nucleus, a midpiece with mitochondria and a tail (reviewed in Billard et al., 1990). In many species, testicular sperm need not undergo final maturation to be able to fertilize eggs. Finally, there is the argument of numbers. A 50% reduction in the production of fertile gametes would have significant consequences for females, while males would still retain their reproductive potential, as sperm usually is produced in excess.

The only types of recessive mutations that are likely to affect fertility in DH males are those which are directly related to critical checkpoints in male meiosis. Such mutations have been identified in yeast and mice and are remarkably conserved in vertebrates (Inoue et al., 1999; Libby et al., 2003). Most of these mutations would produce thread-like (filliform) gonads. Filliform gonads are common in DH progeny and are usually scored as sterile on visual inspection; more histological studies are needed to identify the causes of sterility. We predict that some of these gonads of DH fishes will show arrested stages of spermatogenesis (or oogenesis) as a result of expressions of mutations affecting (fe)male meiosis.

2.7. Homozygous clones and F_1 hybrids

In Table 2, a summary is given of species for which homozygous clones and/or F_1 hybrids have been produced. Given the problems with survival in general, and fertility of females especially, it is not surprising that only a few laboratories have succeeded in making homozygous clones. The majority of the clonal lines listed here are female clone lines. Individual ("first generation") DH females can be reproduced by meiotic gynogenesis. This method of reproduction is easier to perform than mitotic inhibition and the yields of clonal fry are usually much higher. Homozygous clones of females can also be maintained by intra-clone crossing of females with hormonally sex-reversed male sibs.

To our knowledge, there are currently only two species for which androgenetic clones have been produced, rainbow trout and common carp. Fertility usually is not a problem, but DH males can be reproduced only by androgenesis to produce homozygous male clones and yields are typically low. Sex reversal of males to females is possible, but difficult, and has only been achieved in a few species (reviewed in Devlin and Nagahama, 2002).

By crossing two non-identical DH animals, heterozygous clones or F_1 hybrids are produced (Streisinger et al., 1981). This type of normal reproduction usually poses fewer problems. F_1 hybrids are free of recessive lethals and often show hybrid vigour relative to homozygotes in terms of viability. F_1 hybrid animals can be reproduced by androgenesis or gynogenesis to produce *recombined* DH progeny groups (rDH; Fig. 2). Each animal in such an rDH progeny group can be propagated to produce a new homozygous clone. These are commonly called recombinant inbred lines (as in plants: RIL) or strains (as in mice: RIS). To our knowledge, no experiments have been performed in which the viability of rDH has been compared with that of DH progeny of parents from the founder population. It seems probable that recombinant clonal lines with superior reproductive traits can be developed. We recommend that such experiments be conducted, as they will have a high power in detecting and mapping novel QTL relating to embryo- and early larval mortality.

2.7.1. Zebrafish

In zebrafish, the majority of clonal lines originally produced by the Streisinger group were male. Propagation

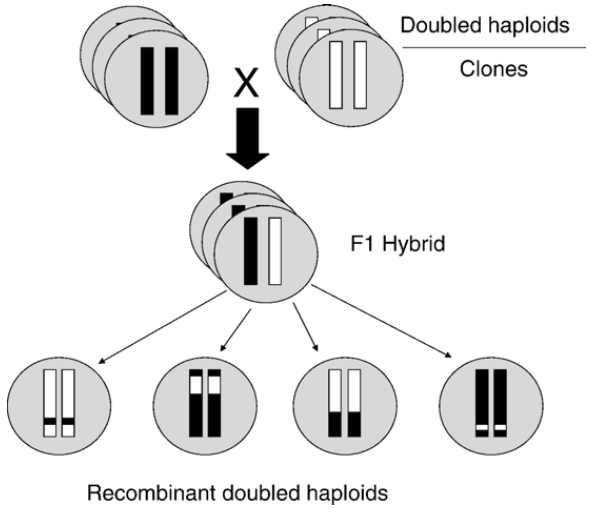

Fig. 2. Production of homozygous clones, F1 hybrids and recombinant doubled haploid (rDH) strains. Legend: Doubled haploids can be reproduced by gynogenesis or androgenesis to produce homozygous clones. Crossing of two DH animals produces groups of genetically identical heterozygous fish: F_1 hybrids. F_1 hybrids can be reproduced by andro/gynogenesis to produce recombinant doubled haploids.

was difficult as these lines do not respond to estradiol treatment to induce male-to-female sex reversal. In 1995, it was discovered that one of the original "Streisinger" female clone lines, "C32", was heterozygous at the *sMda-A* locus (Buth et al., 1995). The line had been maintained by 6 cycles of gynogenesis and intermittent sib matings since 1978. The authors assumed that the mutation had newly arisen, and they estimated that mutation rates were sufficiently high in zebrafish to generate new variation within a few generations of sib-mating. However, the analysis of genetic polymorphism in the C32 strain was repeated 4 years later by Nechiporuk et al. (1999), who discovered that the C32 genome contained a number of randomly dispersed small blocks of closely-linked, heterozygous CA repeats. This contradicts the hypothesis of Buth et al. (1995) that heterozygosity in this line arose by *de novo* mutations. The current status of the C32 line is uncertain. A substrain C32-bc9 was maintained and outcrossed with another inbred strain (SJD) to improve its viability (http://zfin.org/zf_info/dbase/db.html).

2.7.2. Common carp

In the common carp, several clonal lines have been produced. The original clonal lines described in Komen et al. (1991) originated from a single crossbred granddam. To date, one female clone ("E4") and two male clone lines ("E5"; "E7") survive from this group. The male clone lines are XX, homozygous for a recessive mutant at a sex-determining gene, and show low cortisol levels after stress, caused by 17á-hydroxylase deficiency (Ruane et al., 2005). Other clonal lines were later produced by gynogenesis and androgenesis from an F_1 cross of two inbred carp strains from Poland ("R3") and Hungary ("R8"). Initially, seven female clonal lines and two male clones (YY) were produced (Bongers et al., 1997b). The female lines had been selected for their immune response against a synthetic antigen (Wiegertjes et al., 1996). Two of these lines (R3R8-69/45 and R3R8-69/13) and the male lines were confirmed to be homozygous after DNA fingerprinting and skin transplantations and are now third and fourth generation respectively. The other female lines were partly heterozygous and appear to have originated from meiotic diploid females; these were culled. Female clone E4 has poor ovulation response and poor fertility due to spontaneous activation of eggs. It is propagated by crossing with methyl-testosterone-treated E4 males. The R3R8-69/45 clone has good fertility. All male clonal lines are maintained by androgenesis. For androgenetic reproduction, eggs from an E4xE5 F_1 hybrid female are used. These females are highly uniform in egg production and egg quality, with hatching rates from control (normal) fertilizations consistently close to 100%.

Recently, another homozygous clone line has been developed by gynogenesis from the Israeli Dor-70 strain (Ben-Dom et al., 2001). The Dor-70 strain was selected for growth rate and general combining ability between 1965 and 1970 (Moav and Wohlfart, 1976; Wohlfarth et al., 1980).

2.7.3. Rainbow trout

Thorgaard and co-workers used the natural geographic diversity within the rainbow trout species as a source of genetic variation for the production of clonal lines. Rainbow trout are naturally distributed along the Pacific coast of North America from northern Mexico to Alaska, and on the Kamchatka Peninsula of Russia (MacCrimmon, 1971). At this point, nine lines are being maintained. The OSU (Oregon State University) line is a female (XX) line, and has been of primary importance as the line to which various male lines are crossed. The OSU line was derived from a strain which historically was propagated at the Mt. Shasta hatchery of the California Department of Fish and Game. The male lines (YY) are derived from single individuals, taken from domesticated (Arlee and Hot Creek) and (semi-)wild rainbow trout populations (Clearwater (Idaho, USA), Klamath (Oregon, USA), Swanson River (Alaska, USA), Whale Rock (California, USA) and Skookumchuck (Washington, USA). Two Whale Rock (WR) lines have been produced derived from a single male by androgenesis. In addition to the male WR line which is being propagated by androgenesis, a female WR line is being propagated by gynogenesis. The OSU, Arlee and Hot Creek lines have longer histories of rearing in hatcheries than do the Swanson, Clearwater, Klamath, Skookumchuck and Whale Rock lines. The homozygosity of these lines has been confirmed by DNA fingerprinting (e.g., Young et al., 1996; Robison et al., 1999). Gynogenetic lines are not maintained. A single female line, obtained by gynogenesis, was found to be heterozygous after screening with DNA markers (Young et al., 1996).

2.7.4. Nile tilapia

All Nile tilapia clones are derived from a base population that originates from Lake Manzala, Egypt, and which is being kept at Stirling University (UK). Fish from this population subsequently were moved to Göttingen University, Germany. Six homozygous gynogenetic all-female clonal lines were originally developed in Göttingen. These clones were produced from a group of 10 fertile DH females. Five of these clonal lines originate from the same granddam while the sixth has a different genetic background. All clones were confirmed homozygous, based on DNA fingerprinting. Their viability is

highly variable (Müller-Belecke and Hörstgen-Schwark, 2000). Survival at first-feeding varies between 1 and 20% among the clones in comparison to about 45% in controls. Juvenile growth is comparable to controls. In recent years, 2 lines were lost, while two other clones were so difficult to reproduce that it made no sense to perform further experiments with them (G. Hörstgen Schwark, Gottingen University, Germany, pers. comm.). The two remaining lines result from two different *O. niloticus* grandmothers from the Lake Manzala population. One clone has significantly smaller eggs than the other or than outbred tilapia. Both clones still have low viability (first-feeding rates of around 10%; CV 130–170%). Clone crosses (I females × II males) show much better performance and have very stable reproductive parameters compared to outbred controls (Müller-Belecke, 2005). In Stirling, 5 more gynogenetic clones are being maintained. Their homozygosity was confirmed by microsatellite analysis (Ezaz et al., 2004b). Their fertility appears to be less than that of the females from which they were derived. The length of the reproductive period is also reduced (D. Penman, Stirling University, U.K., pers. comm.). Clones are maintained by crossing sex-reversed males with female siblings or by back-crossing to the DH founder female.

2.7.5. Other species

In Japan, two clonal lines were first produced for medaka (*Oryzias latipes*) in 1985 by Naruse et al. (1985). These clones were female, produced by gynogenesis and maintained by mating clonal females with methyltestosterone-treated male sibs. The clonal lines were derived from the orange–red variety and were similar to the classical inbred strains Hd-rR and HNI. These two inbred strains became the most widely used inbred strains in medaka research (Naruse et al., 2004). However, the clone lines were subsequently lost (K. Naruse, Tokyo University, Japan, pers. comm.).

The ayu (*Plecoglossus altivelus*) was cloned by Taniguchi et al. (1994). They subsequently were used in growth and physiological studies (Valle et al., 1994). The ayu clones were discontinued after the end of the project in 1997 (N. Taniguchi, Sendai University, Japan, pers. comm.).

Several homozygous gynogenetic clone lines of Japanese flounder (*Paralichthys olivaceus*) currently are propagated for use in research and aquaculture (reviewed in Arai, 2001). They are being used in studies on sex determination and development. The fertility of these lines is sufficiently good to allow their maintenance (Yamamoto, 1999).

The first clones of amago salmon (*Oncorhynchus masou*) were produced in 1994 by Kobayashi and coworkers. Initially, 6 gynogenetic DH females from two different strains were reproduced by meiotic gynogenesis, but only 2 of these (F01 and F06) produced sufficient numbers of swim-up fry. The survival rate up to 150 days post-fertilization was more than 80%. Isogenicity was confirmed by tissue transplantation (Kobayashi et al., 1994).

2.8. Performance of clones and F_1 hybrids

Homozygous animals show inbreeding depression, and the performance of homozygous clones in terms of viability and fertility usually follows that of the parent from which they were derived (additive genetic relationship is 1, see below). In F_1 hybrids, on the other hand, heterozygosity is restored and although hatching rates may be low due to maternal effects, these animals typically show improved viability compared to clones. Homozygous and F_1 hybrid clones still show phenotypic variation, although the magnitude differs. Phenotypic variation can be partitioned into genetic and environmental variation. Sources of genetic variation are additive variation, variation caused by dominance or epistatic interactions, and variation caused by maternal differences. Non-genetic sources of variation usually are quantified as "environmental variance (V_e)" and "genotype-by-environment interactions (G × E)". As animals from the same homozygous clone or F_1 hybrid are genetically identical, they no longer vary due to genetic factors and all variation observed should be environmental.

To correct for scaling effects, the variation often is expressed as variation relative to the mean or coefficient of variation (CV). In a classical study with inbred strains of mice, Festing (1976) demonstrated that homozygous strains and F_1 hybrids show reduced CV for mandible length compared to outbred or F_2 progeny. A few studies have made a comparison between homozygous clones and outbred control groups of fishes in terms of variation for a variety of traits. In the amago salmon, ayu and Nile tilapia, the phenotypic variation for body weight and length was somewhat reduced in homozygous clones compared to crosses of outbred fish (Kobayashi et al., 1994; Taniguchi et al., 1994; Müller-Belecke and Hörstgen-Schwark, 2000). Interestingly, in tilapia, the difference in variation was consistent over a range of stocking densities, and tended to decrease with higher densities (Müller-Belecke and Hörstgen-Schwark, 2000). The authors suggested that homozygous fish might show less (density dependent) antagonistic behavior towards genetically identical sibs. In common carp, variation in body weight and length was significantly increased in homozygous clones compared to crosses of homozygous animals with outbred sires. However, variation was

comparable to outbred crosses when homozygous clones were produced by mating females with sex-reversed sibs. This indicated that part of the variation in homozygous clones was caused by treatment effects. The reason for the difference between common carp on one hand, and tilapia and ayu on the other is not clear. Survival of clonal carp is high (50–80%) compared to tilapia (max 9%: Müller-Belecke and Hörstgen-Schwark, 2000). A higher survival rate could result in an increased variation in the clonal population if the smaller, deformed animals also survive.

2.8.1. Developmental instability and phenotypic variation

When comparing phenotypic variation in homozygous clones and F_1 hybrids, different sources of environmental variation have to be considered. First, homozygous populations are in general more susceptible to "true" V_e, while F_1 hybrids are less sensitive (Falconer and Mackay, 1989). Second, homeostasis reduces as the buffering of developmental processes against environmental and physiological sources of variability decreases with increasing homozygosity (Lerner, 1954). This reduced homeostasis could lead to developmental instability (DI). DI is measured by comparing bilateral symmetric characters within the same individual (fluctuating asymmetry, or FA) and can be considered an unbiased estimate of deformity. Fluctuating asymmetry is defined as the difference in the expression of a trait between the right side and left side of an animal, and should show a normal distribution with a mean of zero. Traits that exhibit FA typically have high heritability and low CV. Asymmetry itself has low heritability (Leary et al., 1985). Several studies have demonstrated a positive correlation between increasing levels of DI, heterozygosity and fitness (e.g. rainbow trout (Leary et al., 1983) and topminnow (Quattro and Vrijenhoek, 1989)). However, negative correlations also have been reported (reviewed in Palmer and Strobeck, 1986).

Two studies have been conducted to investigate the relationship between DI and homozygosity in DH clones and F_1 hybrids. In the first study, three homozygous clonal lines of rainbow trout, produced by androgenesis, were compared with three F_1 hybrid crosses for fluctuating asymmetry in numbers of pectoral fin rays, pelvic fin rays and gill rakers on the upper and lower arch, respectively (Young et al., 1995). Control groups consisted of crosses of clonal males with outbred females. Overall, the degree of FA was significantly higher in homozygous clones. There was no difference between F_1 hybrids and control groups. The number of pelvic fin rays was the least variable character, the number of gill rakers the highest. The authors also analyzed spotting patterns, as each F_1 hybrid strain showed a distinctive spotting pattern. However, there was no difference in FA for spotting between homozygous fish and F_1 hybrids, suggesting a low heritable component for the trait (Young et al., 1995).

In the second study, DH clonal common carp were compared for variation in length and body weight and for asymmetry in four metric indices relating to cranial dimensions and the number of pelvic fin rays (Bongers et al., 1997a). In a first experiment, four crosses of a DH female with different males were compared. The four isogenic crosses differed in homozygosity from 0 to 0.99, but no difference in variation for body weight or FA was observed. In a second experiment, embryonic damage due to treatment effects was included as a variable. F_1 hybrid females were propagated by gynogenesis to produce meiotic diploid and mitotic DH progeny. Sex reversed males from the same F_1 hybrid were used to produce androgenetic DH progeny. The control group was a normal fertilization between an F_1 hybrid male and an F_1 hybrid female. This time, both the phenotypic variation for length and body weight, as well as the amount of FA increased significantly in all manipulated groups (meiotic diploids and DH) compared to the control group. Values from the control group did not differ from those observed in the first experiment. There were no differences between androgenetic and gynogenetic progeny groups. Taken together, these results show conclusively that temperature treatments are the main cause of increased FA (Bongers et al., 1997a).

3. Applications of doubled haploids and clones

Research with clonal fishes clearly has tremendous potential. Genetic uniformity allows for comparisons on the same genotype over time and under different ambient conditions. This allows estimation of genetic correlations and detection of genotype-by-environment interactions and phenotypic plasticity for complex traits such as sex and gonadal differentiation, stress response, and disease resistance (Bongers et al., 1998).

It is important to note that clonal lines represent single haploid genomes extracted from the population from which they were derived. Caution is warranted in extrapolating results to make broad inferences about the source populations, because a clonal line may not be truly representative of the population from which it was derived. Nevertheless, studies with rainbow trout have shown consistencies between the clonal line and donor population for such traits as development rate (Robison and Thorgaard, 2004) and resistance to *Ceratomyxa shasta* (Nichols et al., 2003). It is possible that genes with large effects on traits which are under significant selection are more likely to become fixed for different alleles among

natural and cultured populations. But, at a more subtle level, fixation of alleles affecting parasite or disease resistance is unlikely, given the ever-changing selective environment faced by (natural) populations. This may increase the likelihood that clonal lines developed from a single haploid genome are indeed representative of their source populations.

Production of uniform, homozygous experimental material is particularly advantageous for many genetic mapping and genome sequencing studies in which interpretations are facilitated by homozygosity. The analysis of genetic polymorphisms (microsatellites; single nucleotide polymorphisms or SNP) is greatly simplified when heterozygotes are absent. Large-scale BAC (bacterial artificial chromosome) fingerprinting and sequencing can benefit when variation due to allelic heterozygosity is eliminated by using homozygous clonal material. Studying molecular variations using approaches such as microarray analyses in advanced backcross/congenic populations segregating for a quantitative trait locus (QTL) will likely become a fruitful approach for dissecting molecular mechanisms underlying the trait of interest (Jansen and Nap, 2001; Wayne and McIntyre, 2002).

Another promising avenue of research lies in development of lines for studying physiological effects of mitochondrial variation. When androgenesis is utilized, there is the potential to produce individuals with identical nuclear genotypes, but which vary in their mitochondrial genotype (Bercsenyi et al., 1998; Brown and Thorgaard, 2002). Brown and Thorgaard (2002) exploited this approach to produce lines of rainbow trout which are identical or near-identical in their nuclear genome but which differ in their mitochondrial haplotype. Bercsenyi et al. (1998) produced androgenetic DH goldfish from irradiated common carp eggs. The hybrid progeny had inherited the nuclear genotype from the goldfish and the mitochondria from the carp. These hybrids will be useful for dissecting the significance of mitochondrial haplotype variation for development, physiological functioning and evolution of species (Bercsenyi et al., 1998). Brown et al. (2006) found that differences in development rate among rainbow trout from one clonal line potentially could be related to variations in mitochondrial type following androgenesis.

Finally, DHs and clones can be used for the analysis of epistatic interactions and estimation of genetic correlations, and, most importantly, for the detection of QTL (Fig. 3). Markers for QTL related to "difficult traits", such as meat quality and disease resistance, are needed to execute marker-assisted selection (MAS), marker-assisted introgression (MAI) and marker-assisted differentiation (MAD) breeding programmes. Crosses between DH animals also can be used to investigate sources of non-additive genetic variation, such as dominance and epistatic variation.

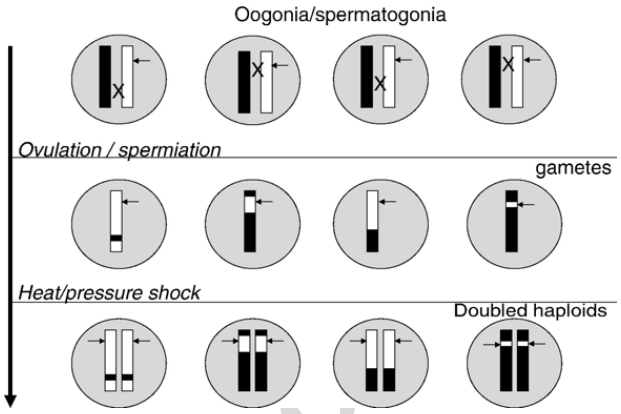

Fig. 3. QTL mapping in doubled haploids. Legend: Each oogonium (in gynogenesis) or spermatogonium (in androgenesis) experiences unique recombination events. Distributions of alleles in DH progeny groups show Mendelian sampling ratios and linkage can be detected by co-segregation of QTL-alleles (arrow) with markers. Only markers which are close to the gene of interest will remain in linkage disequilibrium. Power of detection is enhanced by the absence of heterozygotes.

3.1. Selective breeding with doubled haploids

Doubled haploid animals present some unique genetic relationships which are only found in (animal) somatic clones and twins. The coefficient of coancestry (f_{xy}) between two animals is defined as the chance that a randomly drawn allele *i* from animal X is identical-by-descent to a randomly drawn allele *j* of animal Y. For the relationship between a parent and its DH offspring, this chance is 1/2. The additive genetic relationship "*a*" is defined as 2× the coefficient of coancestry. Thus, the additive genetic relationship between a parent and its DH offspring equals 1. The additive genetic relationship between two DH animals from the same parent likewise equals 1. This means that the breeding value of a parent can be directly estimated from the mean value of its DH progeny. These relationships are based on the assumptions that dominance effects and epistatic effects are negligible. It is also assumed that the environmental variation V_e in DH progeny follows a normal distribution with a mean of zero. However, as discussed previously, this is probably not the case.

Bijma et al. (1997) used these relationships to derive general formulas for estimating breeding values and variance components using DH progeny. They then compared different designs for the accuracy by which the heritability could be estimated. They concluded that

DH designs were more efficient and required fewer animals than full-sib designs when the heritability was low. For high heritabilities, there was no advantage.

The main problem with these studies remains the reliability of the phenotype. As outlined earlier, embryonic damage and the segregation of recessive lethal mutations are major sources of increased environmental variance; this variance is most likely not normally distributed. We therefore do not recommend direct heritability estimates on DH progeny. However, DH animals produce normal gametes and our experiments have repeatedly shown that progeny derived from DH animals show less variation compared to outbred progeny groups. This offers some interesting possibilities in terms of estimating dominance and epistatic effects.

When animals are mated, they transfer their additive genetic value to their offspring. However, there is always an element of uncertainty, as it is not known which alleles are being transmitted to the progeny. This uncertainty is called the Mendelian sampling error. When doubled haploid animals are used as sires, there is no Mendelian sampling error. By crossing various DH animals in a diallel setting, general and specific combining abilities can be estimated. F_1 hybrids also can be used to obtain a series of observations on phenotypic expressions by the same genotype, e.g. meat quality and disease resistance. This approach allows for the detection of (un)favorable genetic correlations between traits.

The theory, and the examples given below, all refer to estimation of genetic parameters using DH animals. The question remains whether DH animals and clones can be used in selective breeding. Selection requires variation and obviously, DH animals and clones represent a dead end in this respect. There are however a number of interesting advantages when DH animals are used in a multiplier as parents of the final product. First, lack of genetic variation can be considered as a form of genetic protection. Secondly, lack of variation could produce increased product uniformity. And finally, the use of homozygous DH males and sex reversed neo-males allows for the production of all-male and all-female stocks. Such stocks can have added value when only one sex has desirable product characteristics.

3.1.1. Examples from common carp

Initial studies with common carp focused on the use of DH to estimate heritability for male and female fertility traits such as GSI, egg size and fertility of eggs. Using the guidelines developed by Bijma et al. (1997), five gynogenetic DH families were produced from different females from a cross between two inbred carp strains R3 and R8 (Bongers et al., 1997b). Animals were randomly sampled at four different ages to assess gonad development. Ovulation was induced by hormonal treatment. Stripped eggs were fertilized with a mixed-sperm sample from three homozygous (DH) males to assess egg quality. Gonad weight, GSI and % normal eggs (fertility) had high heritability (>0.7). Length, weight and egg size had moderate heritability (0.4–0.6). Genetic correlations between egg quality measured at different ages, were high, especially in older DH females. This indicates that DH females can be selected for age-at-maturation and egg quality.

In a follow-up study the genetic basis of age-at-maturation for testis development was studied (Bongers et al., 1997c). Age-at-maturation is a difficult trait to quantify as it requires repeated sampling of the same individual. In such a situation, F_1 hybrids are the ideal tools to dissect additive and dominance effects on this trait. Three DH females were mated with four androgenetic DH males to produce twelve F_1 hybrid crosses, which were sampled over a period of 140 days (100–240 days) to assess testis development. Analysis showed that testis development mainly was controlled by additive genetic effects, with large contributions from the females. Early onset of spermatogenesis coincided with high testis-somatic index (TSI) and late onset with low TSI. The difference between the earliest and latest cross was 130 days.

Based on this information, a synthetic three-way cross was made to serve as standard strain for subsequent studies on spermatogenesis and sex differentiation. This standard strain, denoted "STD" is a cross of an F_1 hybrid female (E4 × E5) with an androgenetic male (R3R8-YY). E4 and E5 are two DH clones derived from the same granddam, and share 50% of the alleles. Spermatogenesis is highly uniform in this strain. Meiosis starts between 80 and 90 days post-hatch (at 25 °C), and first sperm is observed on day 100. Over the years, this strain has been successfully used to investigate the endocrine regulation of spermatogenesis (Timmermans et al., 1997), the effects of xeno-estrogens on testis development (Gimeno et al., 1996, 1997, 1998) and the effects of stress on age-at-maturation (Consten et al., 2001).

A second line of research focused on the stress response of common carp. This time, a large-scale selection experiment was designed using androgenetic DH from semi-wild parents (Tanck et al., 2001b). The design started with crossing six wild sires (Tanck et al., 2000) with homozygous female clone E4. Randomly-picked males from the resulting six half-sib families were reproduced by androgenesis to produce 33 DH families (512 DH animals in total). Heritability estimates for stress-induced plasma cortisol levels were high (0.37–

0.9) indicating good prospects for selection. DH progeny also were screened with 11 microsatellites to confirm homozygosity and to check for possible segregation distortion. This screening revealed two suggestive linkages between a marker and glucose and cortisol levels respectively (Tanck et al., 2001a).

3.2. Genetic and QTL mapping with doubled haploids

As explained in the Introduction, meiotic diploids are produced when the second meiotic division and/or extrusion of the second polar body is inhibited. Genetically, they resemble half-tetrads: the diploid chromosome set consists of maternal sister chromatid pairs (Fig. 1). Depending on the amount of recombination that took place, the individuals are more or less homozygous. Because of this feature, meiotic diploids can be used for gene-centromere mapping and ordering of linkage groups (Streisinger et al., 1981). DH progeny from a single parent on the other hand are fully homozygous since the diploid set of chromosomes arose from duplication of the original maternal or paternal haploid chromosome set. They are not genetically identical. Each individual arises from a single meiotic event and therefore represents a unique sperm or egg haplotype (Fig. 3). In theory, this makes DH animals the perfect tool for genetic mapping and QTL analysis (Picard et al., 1994).

In many species, recombination rates are repressed in males, as compared to females. In vertebrates, it has been assumed that the reduction in recombination was only observed in the heterogametic (XY) sex. While in (placental) mammals this is generally the case, in birds and lower vertebrates this rule no longer applies. In medaka, recombination is suppressed in both XY and XX males. In Japanese flounder (hirame), males have higher recombination rates than females. In rainbow trout the female-to-male recombination ratio is 3.25 to 1 (Sakamoto et al., 2000). In zebrafish, the recombination rate in male meiosis also is dramatically suppressed relative to that of female meiosis, especially near the centromere (Singer et al., 2002). Knowing the rate of recombination in males and females has practical significance for QTL mapping designs. Low recombination rates are useful for the initial approximate detection and mapping of QTL. Furthermore, a system with low recombination tends to maintain linked alleles in *cis*, which is useful for studies on epistatic interaction between linked mutations or QTL. High recombination is needed to distinguish between closely-linked markers and for fine mapping. Clearly the use of androgenesis and gynogenesis offers big advantages in this respect. Androgenesis following recombination in males can be used for the initial detection of QTL, while meiotic diploids produced following recombination in females can be used for gene-centromere mapping and fine-mapping of loci (Young et al., 1998).

3.2.1. Examples from rainbow trout

In this section, we summarize QTL mapping experiments that have utilized DH progeny of rainbow trout. The strategy for detecting QTL follows three steps:

The first step is to detect phenotypic variations among the clonal lines by direct study of the clones, or by comparing the characteristics in crosses to common outbred individuals (Robison et al., 1999; Nichols et al., 2003). The numbers used in the comparative studies involving pure clones are generally not high, as it is difficult to propagate clonal individuals. However, it appears that for many traits, meaningful differences can be detected by using sperm from clonal males to fertilize eggs from common, outbred females. Differences among the lines for traits which are recessive in nature might not be detected with such a design, but traits showing predominantly additive or dominant inheritance should be detectable. Crosses to outbred individuals have the further advantage that groups derived from different homozygous sires can be compared directly and efficiently. By utilizing common eggs, maternal effects are minimized, and the lots being compared can be reared at the same time and under common conditions.

The second step is to analyze segregation of the variation among the DH progeny produced by androgenesis from hybrids. Robison et al. (2001) first utilized doubled haploids for QTL analysis in a cross of two clonal lines to examine the genetic control of development rate. The principal advantages of this approach are the wide genetic diversity present among the segregating individuals and the potential for scoring efficient, dominant markers such as AFLP in such a cross because all of the progeny are homozygous. Potential disadvantages of scoring phenotypes in doubled haploids are the difficulty of producing sufficient numbers of individuals and the possibility of unusual phenotypic expressions related to homozygosity and embryonic damage.

The third step is the statistical analysis linking phenotypes/traits to markers (e.g., AFLPs or microsatellites) among these doubled haploids and the detection of potential segregating QTL. A number of traits now have been successfully mapped. The OSU X Swanson cross was the first to be used for QTL analysis. A difference in embryonic development rate to hatch was initially found between the OSU and Swanson lines (Robison et al., 1999) and a hybrid clone was produced between the OSU female and Swanson male lines. The original intention was to backcross the OSU X Swanson hybrid clone to the

OSU line and to examine marker and trait segregation in the backcross. However, eggs were not available at the same time as was sperm from the hybrid clone. Therefore, a DH progeny from the hybrid clone was produced by androgenesis. This design was highly successful in detecting a major QTL for development rate in the cross (Robison et al., 2001). Currently a congenic line of rainbow trout is being developed in which the QTL for rapid embryonic development from the Clearwater clonal line is introgressed into the slow-developing OSU line (Sundin et al., 2005). Congenic and advanced backcross lines have proven to be very useful in the genetic dissection of traits in mice (Silver, 1995) and plants (Tanksley and Nelson, 1996). By the third generation of the backcross, the association between markers associated with this QTL and development rate was very notable.

The OSU X Clearwater cross was the second used for QTL analysis. The Clearwater line has a number of trait differences relative to the OSU line and this cross has proved fruitful for a number of studies. Derived from the Clearwater River in Idaho, this line is a representative of the inland form of rainbow trout, which is recognized as a distinct group relative to the more widely-propagated coastal strains (Allendorf and Utter, 1979; Behnke, 1992). The Clearwater line is of anadromous (steelhead) origin, and several QTLs were shown to be associated with differences in meristic elements (Nichols et al., 2004), resistance to *Ceratomyxa shasta* (Nichols et al., 2003) and development rate (Nichols et al., 2007). Differences in smoltification (a trait related to the steelhead life history) are still being characterized.

The OSU X Hot Creek cross was utilized for QTL analysis because of substantial differences in natural-killer-cell-like activity between the lines. A single major QTL of very large effect related to natural killer cell-like activity was localized to one linkage group among doubled haploids from this cross (Zimmerman et al., 2004). Several loci also were found to be associated with a difference in numbers of pyloric caecae between the lines in this cross (Zimmerman et al., 2005).

Resistance to the infectious hematopoietic necrosis virus and behavioral differences related to domestication also show differences among lines (Lucas et al., 2004) but have not yet been analyzed. As these traits are likely to be complex in nature, the use of a progeny test design, in which progeny of DH males crossed to outbred females may show potential to better identify trait values and identify QTL,, is needed. This approach is similar in principle to that used for defining the breeding value of sires in dairy cattle based on the characteristics of their daughters.

Finally, the rainbow trout model appears to be promising for studies of sex chromosome evolution and differentiation. Clonal lines of rainbow trout show differences in Y chromosome structure (Felip et al., 2004; Phillips et al., 2004). These clonal lines have been used in the isolation of Y-linked markers and the study of sex chromosome evolution. Felip et al. (2005) identified a number of AFLP markers linked to the Y chromosome in a study of the OSU X Swanson cross. These markers proved to be useful in characterizing the nature of Y chromosome differences among the lines (Felip et al., 2004).

4. Conclusions

There is clearly great potential for wider use of DH animals and clonal lines in genetic and genomic research in fishes and in fish breeding programs. However, a variety of obstacles related to yield, survival, fertility, quality control and sustained commitment of resources still represent challenges to the approach. Increasing the extremely low yields of doubled haploids in experiments with a variety of fish species is still the biggest challenge after 25 years of research. Heat and pressure shocks are easy to apply, but have wide ranging, undesirable side-effects on embryo development. Clearly, more research and more innovative approaches are needed to augment the yields of doubled haploids in a variety of fish species.

Although the application has moved far beyond that originally visualized by Streisinger for analysis of induced mutations, his foresight in advocating this research approach was nevertheless fundamental to the progress which has been made. Perhaps the biggest lesson is the importance of time, patience and continuity for developing and maintaining clonal lines if this approach is to see more widespread use.

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