Immunogenetics of disease resistance in fish

Promotoren: dr. W.B. van Muiswinkel
hoogleraar in de zoölogische celbiologie
dr. C.J.J. Richter
hoogleraar in de voortplanting van lagere gewervelden
aan de Katholieke Universiteit te Leuven
Co-promotor: dr. R.J.M. Stet
universitair docent in de zoölogische celbiologie

Immunogenetics of disease resistance in fish

Geert F. Wiegertjes

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 1 december 1995
des namiddags te half twee in de aula
van de Landbouwuniversiteit te Wageningen

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Wiegertjes, Geert F.

Immunogenetics of disease resistance in fish / Geert F. Wiegertjes. - [S.l.: s.n.]. - III
Thesis Landbouwuniversiteit Wageningen. - With ref. - With summary in Dutch.
ISBN: 90-5485-463-4
Subject headings: immunogenetics; fish / disease resistance; fish. vissen.

Abstract

Wiegertjes G.F. 1995. Immunogenetics of disease resistance in fish. Doctoral thesis, Wageningen Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

The aim of the work described in this thesis is to investigate the possibility to select for immune responsiveness, and subsequently produce isogenic carp (*Cyprinus carpio* L.) lines, via gynogenesis, that express the trait under selection. If possible, this would allow the repeated production of numerous isogenic fish lines, all selected for different immune parameters, that can be used for immunogenetic studies on disease resistance. As a model, we chose a defined antigenic determinant (dinitrophenyl: DNP), coupled to a carrier molecule (keyhole limpet haemocyanin: KLH) in combination with a reliable read-out system (enzyme-linked immunosorbent assay: ELISA), to divergently select carp for the magnitude of their primary antibody response. The possibility to reproduce both homozygous gynogenetic females and functional males with a high or a low antibody response, resulted in the establishment of a number of F_1 hybrid crosses with high or low immune responsiveness to DNP-KLH. Typically, these isogenic lines showed no within-line genetic variation. Between-line genetic variation in susceptibility to infection with *Trypanoplasma borreli*, a haemoflagellate parasite of carp, was dependent upon the immune response type of the carp lines. Major histocompatibility complex (Mhc) class II β-chain polymorphism could be associated with the immune response types described and, in retrospect, may have contributed to the observed differences in magnitude of immune responsiveness to DNP-KLH, and possibly *T. borreli*.

Printed by: Ponsen & Looijen, Wageningen
Cover design: Wim Valen

Dit onderzoek is financiëel gesteund door de Stichting voor de Technische Wetenschappen (STW), en is begeleid door de Stichting Levenswetenschappen (SLW).

Stellingen

1. Gynogenese opent de mogelijkheid tot een snelle selectie op de antilichaamproduktie bij vissen, en tot een afname in proefdiergebruik.
   dit proefschrift

2. Er is behoefte aan meer fundamentele kennis van de genetische invloeden op de immuunrespons, voordat deze gebruikt kan worden als criterium in selectie programma's ter verbetering van ziekteresistentie bij vissen.
   dit proefschrift, pagina 21

3. Het is onwaarschijnlijk dat algemene ziekteresistentie verbeterd kan worden via selectie op bepaalde Mhc haplotypes.
   L. Andersson, 1994. The role of Mhc polymorphism in disease/parasite resistance.
   In: Proceedings of the 5th world congress on genetics applied to livestock production.

4. Selectie op verbeterde ziekteresistentie wordt wel beschouwd als het werken aan een duurzaam systeem, maar is tevens een typisch technologische oplossing.

5. Het gebruik van gynogenese ten behoeve van het wetenschappelijk onderzoek bij vissen vormt een ethisch dilemma.

6. Onderzoekers dienen het te verwachten dierlijk ongerief zwaarder te laten wegen bij de vaststelling van de ethische toelaatbaarheid van voorgenomen dierproeven.
   F.R. Stafleu, 1994. The ethical acceptability of animal experiments as judged by researchers.
   Proefschrift Universiteit Utrecht.

7. Tekort aan tijd, geld en/of ruimte zijn valide argumenten om te kiezen voor een bepaalde aanpak van een wetenschappelijk probleem, maar worden niet als zodanig (h)erkend.

8. De in dit proefschrift beschreven karperlijnen zijn het neusje van de zalm.

9. Het oplossen van een wetenschappelijk vraagstuk is als het maken van een legpuzzel. Je legt eerst het raamwerk, vervolgens de makkelijkste stukjes.

10. Het overdragen van de familienaam van moeder op dochter redt waarschijnlijk het geslacht 'Wiegertjes'.

Stellingen behorend bij het proefschrift
'Immunogenetics of disease resistance in fish"
van Geert F. Wiegertjes, Wageningen, 1 december 1995.

Voorwoord

Het is zover. Alle woorden zijn zorgvuldig gewogen, en aan 90-grams papier toevertrouwd. Alleen de schuine woorden zijn niet wat zij lijken te zijn. Nu kan ik verder slechts hopen dat deze 316 gram genoeg gewicht in de schaal zal leggen om de balans naar de goede kant te doen doorslaan.

Het zweven tussen AIO-status en die van universitair docent was niet altijd gemakkelijk, maar gaf mij ook de ruimte voor een vrije val van enkele jaren, waarvan ik best heb genoten. Gelukkig was de landing zacht. Nu ik mijn jarenlange afzondering van de buitenwereld heb afgerond, kan ik mij eindelijk terugtrekken uit de vochtige catacomben van het vispaleis, en traditiegetrouw eenieder bedanken die mij met raad en daad heeft bijgestaan. En wie ben ik, om met tradities te breken.

Goede raad is duur, dus die komt van promotoren. Wim, jij was de initiator van deze onderzoekslijn, en ik ben verantwoordelijk geweest voor tenminste één stapel op jouw buro. Bedankt voor het vertrouwen en de vrije hand. Carel, je bent je er waarschijnlijk niet van bewust, maar de discussie van mijn doctoraalscriptie eindigt met de luid ingefluisterde suggestie om het onderzoek voort te zetten met een "selectie van individuele vissen met karakteristieke kenmerken". Bij deze.

Raad en daad. René, ik denk dat wij door de jaren heen de balans goed in evenwicht hebben gekregen en hoop dat dat nog even zo mag blijven. Mijn gegoochel met vissen mag jou dan hoofdbrekens kosten, jouw engelse woordenschat heeft mij al menige vraag opgeleverd. Guus, onze ideëen over de wetenschappelijke invulling van ons bestaan lopen niet ver uiteen. Niet alleen waren wij de vleesgeworden WIAS-gedachte, onze vergaderuurtjes verenigden het nuttige met het aangename.

In volgorde van natuurlijk verloop, natuurlijk alle studenten die, in welke vorm dan ook, hun steentje hebben bijgedragen; Jürgen, Sunil, William, Christel, Astrid, Mark, Paul, Anita, Maarten, Garry, Nuno, Annemieke. En natuurlijk mijn paranimfen. Adrie, op onze reis naar het Polen hebben wij een muur geslecht. Verder ben jij de enige geweest die er altijd vertrouwen in had dat ik het wel significant zou krijgen. Paul, de wereld der alpenhutten is een kleine. Wie had ooit gedacht dat jij mij zou inwijden in de beginselen van de geheimzinnige wereld der DNA-bouwstenen. Ik hoop dat jullie beiden mij ook op het podium bij zullen staan.

Natuurlijk als laatste en als beste, de familie. Vader en moeder, ik hoop dat jullie nu beseffen dat promovendi ook maar mensen zijn. Bedankt voor het nooit aflatende vertrouwen. Lieve Imke, we hebben gezien dat het 'droge Noorden' en het 'uitbundige Zuiden' een prachtige vorm van heterosis geeft. Wederom door dik en dun. En wat die tijd betreft, dat lukt ons wel.

Geert

Contents

Chapter 1	General introduction	**3**
Chapter 2	Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis	**37**
Chapter 3	Divergent selection for antibody production to produce standard carp (*Cyprinus carpio* L.) lines for the study of disease resistance in fish	**51**
Chapter 4	Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.)	**61**
Chapter 5	Investigations into the ubiquitous nature of high or low immune responsiveness after divergent selection for antibody production in common carp (*Cyprinus carpio* L.)	**73**
Chapter 6	Investigations into the immune responsiveness of F_1 hybrids of homozygous carp (*Cyprinus carpio* L.) selected for high or low antibody production: indications for immune response gene control	**89**
Chapter 7	General discussion	**113**
Summary		**127**
Samenvatting		**133**
Curriculum vitae		**141**

Chapter 1

General introduction

§1.1 Genetic influences on disease resistance or susceptibility

Unless one is dealing with inbred animals, owing to genetic heterogeneity, host populations will inevitably show a variability in response to infection with a pathogen, which will be reflected in variability within parameters for natural resistance. It is however acquired immunity by which the host can pre-eminently influence the outcome of infections. The specificity of the acquired immune response is determined by the major histocompatibility complex (Mhc) at the surface of the antigen presenting cell (APC), the T cell receptor and the immunoglobulin molecule on the surface of the B cell. The amount and isotype of antibody produced, the type and quantity of cytokines produced, and the longevity of the immunological memory are additional factors that affect the quality of immune responsiveness (Doenhoff and Davies, 1991). Genetic factors can influence these protective mechanisms at many stages, although some mechanisms seem to be of particular significance for correlations between variation in immune responsiveness and levels of resistance to infection (Wakelin, 1988; see also Figure 1).

At this point it may be helpful to consider the most crucial steps in the generation of an immune response, which include the processing and presentation of antigen. In general, $CD8^+$ T cells become cytotoxic upon activation and lyse the target, whereas $CD4^+$ T cells exert a helper (T_H) function by secreting lymphokines that may stimulate, for example, B cells in their function. Largely dependent on the route of entry of the antigen into an antigen-presenting cell (APC), often tissue macrophages or B cells, antigen processing will lead to the binding of peptide fragments to one of two classes of major histocompatibility complex molecules. The Mhc is a group of closely linked genes which code for membrane glycoproteins. Mhc class I molecules are expressed on all somatic cells, whereas class II molecules are found mainly on cells of the immune system. The Mhc class II binding peptides are derived from exogenous antigens, while the peptides that bind Mhc class I are generally derived from the cytoplasm. Lymphokine-activated B cells present peptides and interact with T cells, to differentiate into plasma cells or memory B cells. The presentation of antigenic peptides to the T cell is Mhc-restricted.

In most mammalian species, Mhc molecules are highly polymorphic. The selection mechanism favouring extensive polymorphism is not yet clearly understood, but the most common view is that this phenomenon is related to significant influences of allelic Mhc molecules on the efficacy of the immune response and thereby on the resistance to pathogens (Teale, 1994). Although the Mhc may not be the only polymorphic system, it is by far the best described one, and instrumental to the outcome of an immune response.

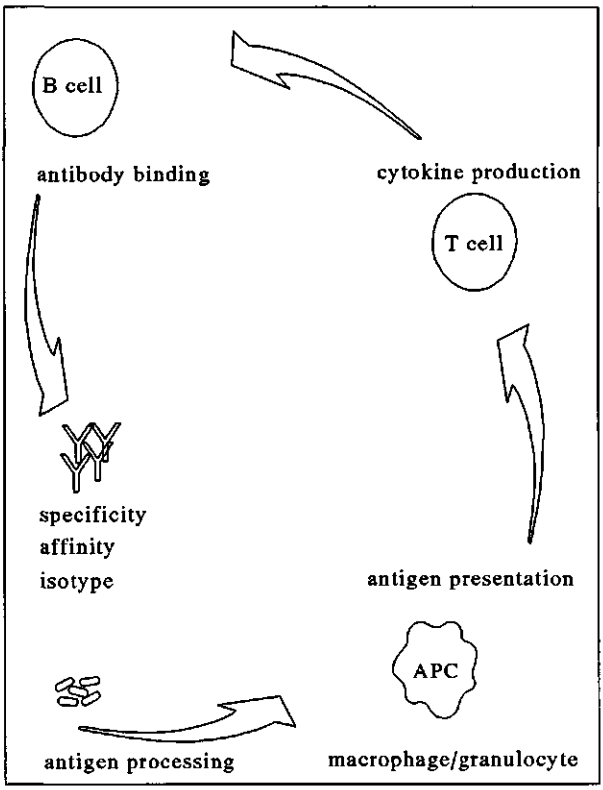


Figure 1. Schematic representation of the generation of acquired immune responsiveness, denoting crucial steps that can be of particular importance for correlations with levels of resistance to infection (modified from Wakelin, 1988).

T cells recognize antigen via a T cell receptor (TCR) associated with the CD3 complex. During the maturation of T cells in the thymus, up to four different elements are incorporated in the gene coding for the heterodimeric TCR-molecule by a process of gene rearrangement. Although probably not all different gene elements have an equal likelihood of being used, the potential for diversity of the TCR is assumed larger than the number of T cells present in the individual. Since T cells recognize antigens only in association with "self" Mhc, consequently, the T cell response will largely be determined by the Mhc type of the animal. This Mhc-restriction is considered unlikely to introduce gaps in the T cell receptor repertoire which can be held responsible for a reduction in immune responsiveness (Doenhoff and Davies, 1991).

Similarly, the genes coding for immunoglobulin molecules on the B cell surface, are generated by gene rearrangement. In addition, somatic mutation gives a further antibody sequence diversity that contributes to an increasing affinity during the course of the immune response. Responses to multi-epitope antigens often utilize numerous V genes and gene families, although these responses may be genetically restricted in some instances (Kofler *et al.*, 1992). In general, the assembly of DNA segments by recombination to form functional genes, however, generates an enormous number of different antibodies and receptors. It is therefore unlikely that genetic differences in disease resistance could be easily explained by the lack of specific antigen receptors.

§1.2 The approach to genetic regulation of disease resistance

Current methods to control disease in livestock, including fish, consist of among others, vaccination, medication and eradication of certain diseases. Improving genetic disease resistance may be another, preventive, measure against infectious diseases, attractive because of its prospects of prolonged protection. In fact, the ability of a host to resist infection with a wide range of viral, bacterial and parasitic pathogens is strongly influenced by genetic factors (Skamene and Pietrangeli, 1991). There are a number of approaches to study the genetic influences on disease resistance in animals. One example is the long-term research strategy aimed at a full genetic analysis of host resistance, such as proposed by Festing and Blackwell (1988). In short, the first phase consists of the development of a set of suitable parameters in order to obtain clear-cut differences in disease resistance between inbred lines. The strategy continues with a study of the response in a large number of inbred lines in order to find a pattern which could be accounted for by a single genetic locus. On establishment of such a pattern, classical crosses, which include F_1 hybrids, F_2 hybrids, and backcrosses to both parental lines, can be used to demonstrate Mendelian inheritance to collect evidence for a single gene mode of inheritance. This can be followed by linkage studies with known genetic markers to map the Mendelian locus on the chromosome. Although this long-term strategy can, in theory, be applied to any given animal species, it is highly dependent upon the availability of inbred lines of the species under investigation. Inbreeding is most often characterized by successive mating of related animals and typically reduces the genetic variation within lines. Inbred lines have an inbreeding coefficient (F) practically equal to one, with F being the probability that two genes at a given locus are identical by descent. Typically, the reduced genetic variation in numerous inbred lines of the mouse, has allowed a detailed laboratory-based analysis of host-parasite relationships (Wakelin, 1988).

A strategy as described at the previous page, can not be used to study genetic aspects of disease resistance in livestock, because of an almost general lack of standardized inbred lines, due to the relatively long generation intervals. The homozygous state of increasing numbers of genes, typical of inbred lines, can result in "inbreeding depression", a reduction of the mean level of characters closely connected with fitness, such as fertility, which is the reason for livestock breeders to avoid inbreeding. Instead, in livestock, the search for associations between disease resistance and marker genes, within the population, is the most common approach. Markers can range from phenotypical features such as colour, to DNA polymorphisms of functional importance such as that of the major histocompatibility complex. In the mouse, the majority of studies have either focused on the selection of lines with a genetically determined response to particular antigens, or on selective breeding for changes in immune responsiveness.

Thus, there are (at least) two approaches to study genetic determination of disease resistance in animals, family selection and selective breeding, which in some aspects, can be considered each others opposites, as depicted in figure 2. The search for associations between disease resistance and marker genes can be a first step towards both approaches. *Family selection* is based upon the characterization of families which exhibit a clearly different response to infection. The use of inbred lines, and of congenic lines (see §1.4.2), has greatly simplified the analysis of such responses. Upon finding a clear pattern of differences in (immune) responses, classical crosses to detect Mendelian segregation, and linkage studies, can finally lead to the identification of the gene. *Selective breeding*, on the other hand, starts with a clearly defined (immune) parameter that could have an effect on resistance to the disease, leading to families exhibiting different patterns of response upon infection with the pathogenic organism. These families can be the desired "end product" (livestock), or be used for segregation studies as described above. Here, there is no need for inbred lines, because selective breeding aims at families homozygous only for those genes determining the defined parameter. Short generation intervals are highly beneficial to selective breeding, however, because many generations may be needed to attain homozygosity, even for a limited number of genes.

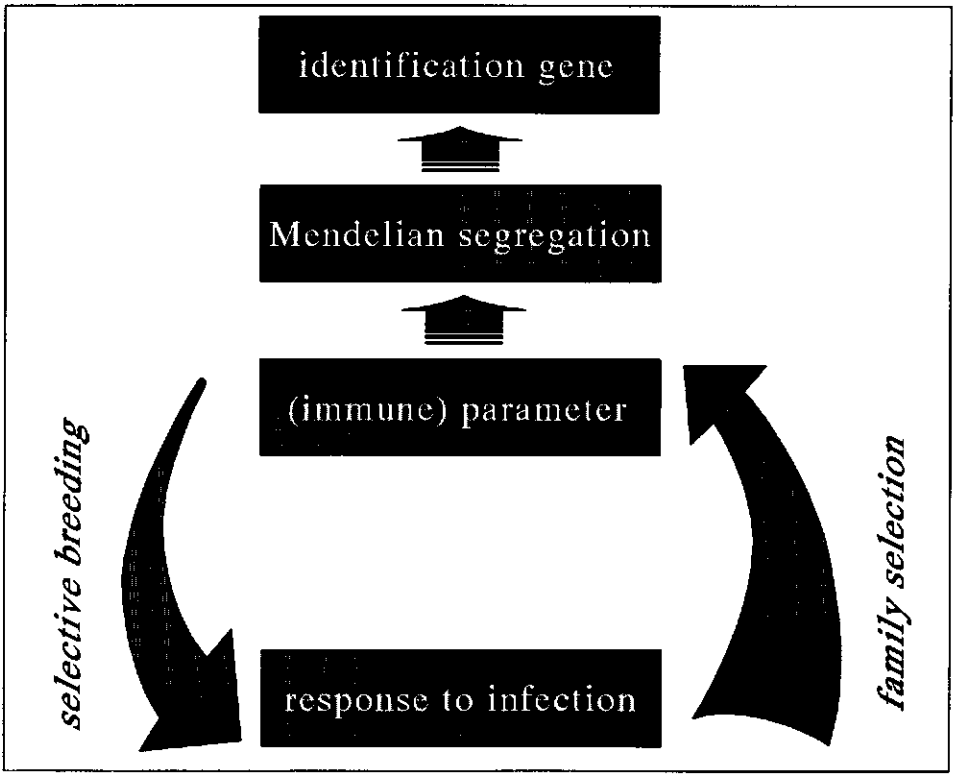


Figure 2. Schematic representation of two major approaches to genetic determination of disease resistance in animals. Characteristic for *family selection* is the narrowing down from the (different patterns of) response to infection, to the identification of the gene responsible for Mendelian segregation. *Selective breeding* can be characterized by an expansion from a relatively defined starting product (for instance specific antibody production), to families exhibiting different patterns of response to infection. These families can then be used for Mendelian segregation studies identifying the gene(s) involved.

§1.3 Selective breeding

§ 1.3.1 Biozzi model

Many studies have focused on modifications of antibody- or cell-mediated immunity by selective breeding. The best known examples of modification of antibody responsiveness are certainly the divergent selections initiated by Biozzi *et al.* (reviewed in 1979) for high and low antibody production to optimal doses of multideterminant antigens such as sheep red blood cells (SRBC) in the mouse (Fig. 3, Table 1). These divergent breedings resulted in lines assumed homozygous for the genes determining antibody responsiveness only. The different antibody response was primarily ascribed to genetic modifications in macrophage metabolism and antigen presentation (Biozzi *et al.*, 1984). The high rate of macrophage metabolism in the low responder lines suggested an inverse relationship between genetic regulation of macrophage activity and antibody responsiveness (Table 1). The strong activity of the low-responder macrophages caused an increased resistance of the low lines to intracellular pathogens, and a decreased resistance to extracellular pathogens, and *vice versa* for the high lines. In spite of their stronger antibody response, high-responder mice were more susceptible to infection with typically intracellular pathogens, e.g. *Salmonella typhimurium* (Biozzi *et al.*, 1984).

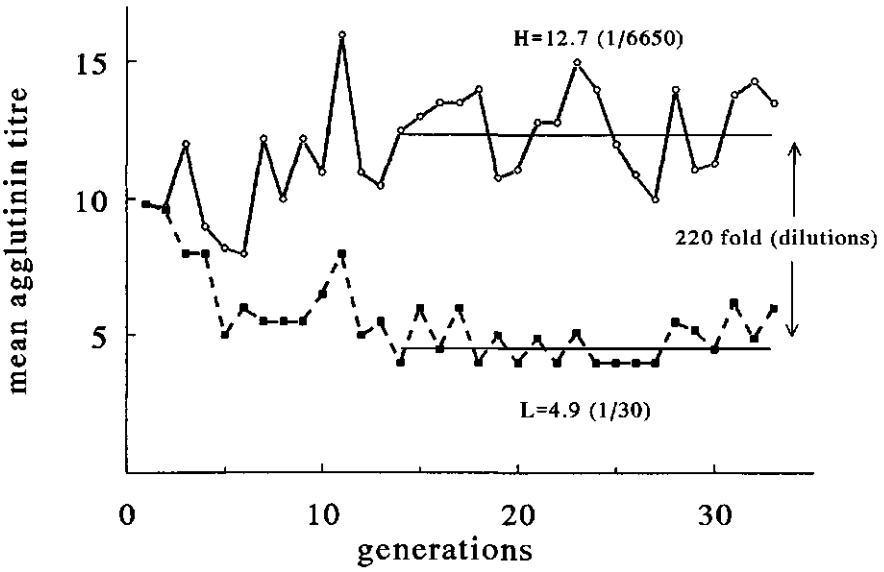


Figure 3. Anti-sheep red blood cell (SRBC) agglutinin response (log 2) at 14 days after immunization, in successive generations of high and low responder mouse lines (selection I). F_0-F_{16}: Divergence of high and low lines. F_{16}-F_{38}: Interline separation (straight line). (modified from Biozzi *et al.*, 1979)

The number of independent loci involved in determining the antibody response was initially determined to be 5 to 10 (Biozzi *et al.*, 1979), but more recently, in a study which reported a positive correlation of life span with antibody responsiveness of these lines, this number was limited to 5 to 7 independent loci (Covelli *et al.*, 1989). Immunization with an optimal dose of SRBC lead to an estimated 10% of the divergence in immune response that could be explained by Mhc (or *H-2* for the mouse) class II differences, which demonstrated the important genetic effect non-Mhc genes can have on the immune response. The use of threshold doses of SRBC, however, resulted in a regulation of the antibody response by only two loci, one of which was the Mhc, as assessed with skin grafting and lymphocytotoxicity assays (Biozzi *et al.*, 1979). Today, the Biozzi mouse lines have been *H-2* typed, and retyped, using polyclonal as well as monoclonal antibodies reacting with known class I and class II determinants (Colombani *et al.*, 1979; Frangoulis *et al.*, 1990; Liu *et al.*, 1993).

Although the selections by Biozzi *et al.* were based on the primary antibody response to multideterminant antigens such as SRBC, the interline differences were shown to be polygenic traits common to several antigens, or pathogens. For example, the Biozzi high- and low-responder mice have been used to investigate whether genetically regulated differences in antibody response to *Trypanosoma cruzi*, the causative agent of Chagas' disease in Central and South America, could alter resistance to infection. *T. cruzi* belongs to the stercorarian trypanosomes for which transmission takes place by contamination through the faeces of the insect vector, after which flagellated trypomastigotes multiply extracellularly in the blood. Antigenic variation has not been observed for stercorarian trypanosomes. The immunosuppression generally associated with *T. cruzi* infection, has recently been ascribed to a trypanosomal immunosuppressive factor that blocks interleukin-2 receptors (Kierszenbaum and Sztein, 1990). The low-responder Biozzi mice were shown to be more susceptible to infection with *T. cruzi* than the high-responder mice. The role of antibody was supported by passive transfer of resistance to low-responder mice by high-responder immune plasma (Kierszenbaum and Howard, 1976). However, the extent to which resistance was directly controlled by one or more of the genes regulating the antibody response was not determined.

Table 1. Summary of the modifications in immunity that resulted from selective breeding for anti-SRBC agglutinin response in the mouse (modified from Biozzi *et al.*, 1984).

	Antibody-mediated immunity	Macrophage metabolic activity	Cell-mediated immunity
High line	+++	+	++
Low line	+	+++	++

Biozzi found the differences in antibody responsiveness after divergent selection to be primarily due to genetic modifications of macrophage function. The lack of differences in T cell activity between the selected lines suggested an independent genetic control of antibody- and cell-mediated immunity (Table 1). This hypothesis was subsequently examined by the setting up of a divergent selection based on the quantitative proliferation (PHA stimulation) of mouse T lymphocytes. Indeed, the selection procedure resulted in differences in T cell responses which were independent from B cell function (Stiffel *et al.*, 1977). The T cell response, similar to the findings above for the B cell response, was found to be under polygenic control. Unstimulated T lymphocytes from the low line were less viable in *in vitro* culture (Stiffel *et al.*, 1983), and it was concluded that this divergent selection had apparently not affected the accessory cell function of the macrophages, but the capacity of the low line lymphocytes to react to stimulatory signals (Stiffel *et al.*, 1987).

§ 1.3.2 Selective breeding in livestock

In the chicken, similar to Biozzi, van der Zijpp and Nieuwland (1986) initiated a divergent selection for antibody responsiveness to SRBC. A substantial genetic influence on the antibody response was calculated based upon the information of nine generations (Pinard *et al.*, 1992). Further studies on subsequent generations of these high and low responder chickens, to identify the mechanisms underlying the divergence in antibody response, revealed no differences with respect to phagocytic activity (Kreukniet *et al.*, 1994b), but did show that the selection had apparently affected the cellular response (Kreukniet *et al.*, 1994a). It was concluded, however, that not phagocytosis, but antigen processing and presentation determined the differences between the chicken lines (Parmentier *et al.*, 1994). The effect of the Mhc, or *B*-complex in the chicken, on the selected differences in immune responsiveness, was investigated in classical F_1 and F_2 crosses. This led to an estimate that only 3.5% of the total variation in antibody titre could be explained by the Mhc (Pinard and van der Zijpp, 1993). Thus, similar to Biozzi's findings in mice, the interline differences were shown to be polygenic traits, with the Mhc being one of the gene (complexes) involved.

To study the response to other antigens, the chicken lines were infected with Marek's Disease (MD) virus, which causes a lymphoproliferative disease. The low line chickens were more susceptible to MD than the high line chickens (Pinard *et al.*, 1993). In contrast, another divergent selection for antibody production to SRBC by Martin *et al.* (1989), resulted in high line chickens that were more susceptible to MD. Later, Martin *et al.* (1990) observed an increase in the proportion of the B^{21} allele in their high antibody line in response to selection. Interactions of Mhc genotypes with background genome and with sex suggested a complex

picture, however, and may explain the differences between this study and previous findings by Briles *et al.* (1977), where the B^{21} allele was associated particularly with resistance to MD.

Often, the most important aim of selective breeding of livestock is the improvement of overall resistance to pathogens. Indirect selection for increased humoral responsiveness to SRBC, such as initiated by van der Zijpp and Nieuwland (1986), however, could not easily achieve an overall improvement of disease resistance (Parmentier *et al.*, 1994). As a consequence, more recently, efforts have been made to enhance disease resistance in chickens by selective breeding based upon a large number of immune parameters. The different protocols that have been used to select poultry strains for immune competence have been summarized by Bacon (1992). One example is the divergent selection based on multitrait immunocompetence (antibody production, carbon clearance, cell-mediated response to PHA), which resulted in a successful alteration of the humoral response after seven generations without compromising cell-mediated immunity (Kean *et al.*, 1994a). In comparison among lines, a positive influence of the B^{21} haplotype on the selection index was found, although likely influenced by a natural outbreak of MD in the base population (Kean *et al.*, 1994b).

Similarly, in pigs, efforts have been made to enhance disease resistance in a broad manner by evaluation of immunocompetence profiles. For this purpose, genetic differences in a large number of immune parameters were estimated for several breeds (Buschmann *et al.*, 1985) or, composite selection indices were calculated (Mallard *et al.*, 1992). In the latter study, a combination of immune parameters was used to divergently select (Yorkshire) pigs for immune responsiveness. Already after one generation of selection, the line differences were most apparent for the most heritable traits; secondary antibody production to hen egg-white lysozyme (HEWL) and ConA stimulated lymphocyte proliferation (Mallard *et al.*, 1992). After three generations, an unchanged monocyte function, as analyzed by superoxide anion production, suggested that the divergent selection for immune responsiveness had not modified phagocyte function (Groves *et al.*, 1993). Also, quantitative Mhc class II expression on the monocyte cell had not changed due to the divergent selection and was thus not responsible for the intergroup differences in immune responsiveness. It was concluded that, apparently, the paradigm derived from the Biozzi experiments that selection for antibody responsiveness indirectly modifies mononuclear phagocyte function, is not necessarily true for pigs selected on the basis of a combination of immune parameters (Groves *et al.*, 1993).

§ 1.4 Family selection

§ 1.4.1 Detection of immune response genes

Immune response (Ir) genes, regulating the antibody response in an antigen-specific manner, were first discovered in the laboratory mouse (McDevitt and Sela, 1965; 1967). They noted, working with branched multichain synthetic polypeptides, that if histidine was substituted for tyrosine, to produce poly (His, Glu)-poly-(DL-Ala)-poly(Lys), or (H-G)-A--L, instead of (T-G)-A--L, the genetic control completely reversed. Today, an immune response gene is defined as conferring non/low responsiveness to a particular antigen on some individuals and (high) responsiveness on others, depending on the Ir gene they carry (Klein, 1986). It is now recognized that Ir gene control is the product of the Mhc class II genes (Schwartz, 1986). In fact, the T cell-dependent response to all polypeptide antigens is under Ir gene control, but analysis of the response to individual antigenic determinants and the use of inbred lines appeared to be key factors in the unmasking of Ir gene control.

§ 1.4.2 Immune response genes in livestock

The chicken has the best-described immune system of all livestock species, likely due to its relatively short generation interval that has allowed the development of inbred lines and lines congenic for the *B*-complex. Characteristically, the *B*-complex has a high degree of similarity with mammalian class II, but not class I genes, and a very low frequency of recombination of class I and class II genes owing to their close proximity on the chromosome (Kaufman *et al.*, 1995). Mhc-linked antibody responses to synthetic polypeptides such as G-T, a copolymer of glutamic acid - tyrosine, have been demonstrated (Koch and Simonsen, 1977).

Recently, eight 15.*B* congenic lines were developed to characterize the influence of several common *B*-haplotypes on resistance to Marek's disease. Seven haplotypes, among which B^{21}, were introduced into a chicken line homozygous for B^{15}. The procedure typically involved crossing an animal (Y) with a certain trait (e.g. the B^{21} haplotype) with an animal (X) that does not express B^{21}, but B^{15}. The offspring, that will have both B^{15} as well as B^{21}, are backcrossed to X, after which the backcross offspring are screened for the Y trait; the B^{21} haplotype, and so forth. With each backcross there is a further dilution of the Y genes, except for the B^{21} haplotype under selection. There is however a progressive enrichment of the X genes, which are now called background genes. Thus, the effects of different *B*-haplotypes could be studied in chicken lines with only one (allelic) difference on the same genetic background. Upon infection of the congenic chicken lines with MD virus, it was found that, depending on prior vaccination and virulence of the virus strain, several haplotypes other than B^{21} could influence resistance to disease (Bacon and Witter, 1992; Schat *et al.*, 1994).

Vaiman *et al.* (1978) detected Ir gene control of antibody production in the pig, after screening with a panel of eight antigens. They found that the immune response to HEWL was genetically controlled by at least one gene linked to the Mhc, or *SLA* complex. This observation was later confirmed for miniature pigs of which lines with defined *SLA* haplotypes are available; lines expressing certain haplotypes had increased antibody reactivity to not only HEWL, but also to (TG)-A--L and SRBC (Mallard *et al.*, 1989).

On investigations of cattle, Lie *et al.* (1986) detected an association between immune responsiveness to human serum albumin, or to (T-G)-A--L, and the bovine Mhc (*BoLa*), but most studies failed to detect a correlation between immune reactivity and *BoLa* (class I) molecules. The lack of inbred lines of cattle for the evaluation of immune responsiveness (Lewin, 1989), and the improved possibilities to detect Mhc class II polymorphism, apparently directed further research on Ir gene control towards an *in vitro* measurement of T cell proliferation. Bovine T cell proliferative responses to ovalbumin were found dependent upon Mhc class II molecules, dividing animals into non-responder and responder groups (Glass *et al.*, 1990). The subsequent development of T cell lines from the responder groups, and detection of Mhc class II polymorphism with one-dimensional isoelectric focusing, demonstrated that the *in vitro* response to ovalbumin was under Mhc restriction (Glass *et al.*, 1991a). A similar division into non-responder and responder T cell lines, related to *BoLa* class II type, was recently described after immunization with synthetic foot-and-mouth disease virus peptides (Glass *et al.*, 1991b).

§ 1.4.3 Genetic regulation of resistance to pathogens in the mouse

A large number of studies have focused on the selection of inbred mouse lines with a genetically determined response to not only particular antigens, but also to pathogens. In relation to pathogenic organisms, as a general rule, non-Mhc genes appear to regulate the (early) effector phase of the immune response. The later phase of elimination of a pathogenic organism often depends on the generation of Mhc-restricted T cell-mediated immunity. Non-Mhc gene control is often more easily detected, likely because this is less affected by extrinsic factors such as parasite numbers than Mhc genes and their effects (Wassom and Kelly, 1990). Where the use of Biozzi high and low responder lines failed to accomplish a clear-cut genetic effect on the resistance to infection with *Trypanosoma cruzi*, the use of mouse lines congenic for *H-2* much simplified the analysis of *T. cruzi* resistance. However, Mhc influence on resistance to infection with *T. cruzi* could only be detected in congenic lines with background genes conferring intermediate resistance. Apparently, depending on the criteria used to monitor the course of infection, strong background genetic influences on resistance or susceptibility may mask the influence of *H-2*, even in congenic lines (reviewed by Blackwell, 1988b).

Although many studies have shown genetic differences between inbred mouse lines in resistance to infection, the studies that also identified the genes and mechanisms regulating the different responses have mostly remained limited to intracellular pathogens of host macrophages (Blackwell, 1988a). One of the best described examples is the regulation of innate resistance, or susceptibility, to infection with several mycobacterium species (e.g. *Mycobacterium bovis*). Here, the outcome of the late phase of infection is associated with genes of the Mhc, but the early phase is controlled by the expression of a single dominant gene on (mouse) chromosome 1, designated *Bcg*. The same gene has been shown to control the natural resistance to infection with antigenically and taxonomically unrelated intracellular bacteria or parasites, such as *Salmonella typhimurium* (*Ity*) and *Leishmania donovani* (protozoa; *Lsh*). It is now well established that the *Bcg-Ity-Lsh* gene is expressed by mature tissue macrophages, and apparently controls an interferon-γ (IFN-γ) activation of cytotoxic macrophages regulating intracellular replication. Macrophages from innately resistant mice are genetically programmed to switch more readily to the activated mode (Schurr *et al.*, 1991). The macrophages, with enhanced cytotoxic or cytostatic activity, appear superior in the expression of surface markers associated with the state of activation (e.g. upregulation of Mhc class II molecules) and in the production of toxic oxygen and nitrogen oxide radicals (Blackwell *et al.*, 1991). The 'run-on' effect of being resistant is the more efficient presentation of antigen to T_H cells that subsequently produce IFN-γ (Wassom and Kelly, 1990). Indeed, phagocytosis or respiratory burst activity of macrophages from resistant mice was found to be superior to that displayed by macrophages from susceptible mice (Schurr *et al.*, 1991). A candidate gene, named the natural resistance-associated macrophage protein gene, or *Nramp*, has recently been identified. RNA expression studies of a genomic interval known to contain the *Bcg-Ity-Lsh* gene, showed that *Nramp* was expressed exclusively in macrophage populations and that susceptibility to infection was associated with a nonconservative substitution within the predicted transmembrane domain 2 of *Nramp* (Vidal *et al.*, 1993).

In (cutaneous) infections with *Leishmania major*, an obligate intracellular parasite transmitted by flies, there is no obvious innate versus acquired phase of resistance (Wassom and Kelly, 1990). *Leishmania* spp. enter cells passively by allowing itself to be phagocytosed into macrophages (Ash, 1991), where they exist only within the phagolysosome. Activation of the macrophage is required for elimination (Locksley and Scott, 1991). In this infection model, there is evidence for a correlation between resistance and cell-mediated immunity, with the function of protective T cells being the production of lymphokines that activate macrophages to resist infection or kill intracellular parasites. In fact, the model system is among the most clear-cut examples of differential regulation by ($CD4^+$ $CD8^-$) T helper (T_H)

subsets. The current hypothesis is a delicate balance between T_H1 and T_H2 cells, of which as a general rule, in case of infection, T_H1 cells and products are host-protective and T_H2 cells promote disease (Mosmann and Moore, 1991). T_H1 cells produce interleukin (Il)-2, IFN-γ and lymphotoxin, and often mediate cell-mediated immunity, recruiting and activating cells such as granulocytes and macrophages to the site of infection. They may deal more efficiently with intracellular pathogens. T_H2 cells produce Il-4, Il-5, Il-6 and Il-10, and induce strong antibody production. They may be more important for dealing with extracellular pathogens (Mosmann and Moore, 1991). T_H1 cells can be even further categorized into sub-populations; macrophages infected with *L. major* induce parasite-protective T_H1 subsets, while uninfected macrophages presenting killed *L. major* select host-protective T_H1 subsets (Ash, 1991).

§ 1.4.4 Genetic regulation of disease resistance in livestock

Often, the approach taken to study genetically determined disease resistance in livestock differs from that in laboratory animals. Disease data, collected under farming conditions, are analyzed to subsequently demonstrate inherent differences in response to infection, followed by an examination for correlations with (immuno)genetic markers. Detailed knowledge about putative genetic markers is often not available, and the search for candidate genes that correlate with disease resistance can only be done on a "best guess" basis as being possibly involved in genetic control. The ways to select livestock for disease resistance have been reviewed extensively (Gavora and Spencer, 1983; Warner *et al.*, 1987; Rothschild, 1991).

Direct breeding for disease resistance is based upon survival after challenge of the breeding stock. Despite several examples of successful direct selection for resistance to specific diseases in livestock species, probably the best known is the selection of chickens resistant to MD, which led to a positive association with the B^{21} allele (Briles *et al.*, 1977).

In light of its pivotal role in the immune response, the Mhc has been a frequent source of candidate genes for disease resistance in livestock species (Teale, 1994). For example, although the detection of relationships between immune responsiveness, or disease resistance, and *BoLa* types, has for long been limited by the lack of class II typing and the relatedness between individuals within and between herds (Lewin, 1989), the supposed linkage with the much better (serologically) defined class I haplotypes initiated a number of correlation studies (summarized by Østergård *et al.*, 1989). More frequently today, the predictive value of class II polymorphisms for the incidence of disease in cattle can be tested. A significant association of certain haplotypes with susceptibility to clinical mastitis has been found (Lundén *et al.*, 1990). However, mastitis is considered a polygenic disease, and indeed, the *BoLa* haplotype was calculated by Mejdell *et al.* (1994) to contribute approximately 5% only to the total genetic variance in susceptibility to mastitis. Class II polymorphism has also been associated

with resistance to bovine leukemia virus, likely caused by amino acid differences in the putative antigen binding residues, which provided a molecular basis for Ir gene control in cattle (Xu *et al.*, 1993). Studies on miniature pig lines with defined *SLA* haplotypes however, have indicated that it is unlikely that general disease resistance can be improved by selective breeding for certain *SLA* haplotypes (Lacey *et al.*, 1989). The functionally relevant polymorphism of the Mhc seems to be maintained by some form of balancing selection; no preferred allelic form appears to exist in the population (Andersson, 1994).

Indirect breeding for disease resistance is characterized by a selection for markers, not necessarily influencing the trait under investigation, or by a selection for candidate genes. Indirect breeding often directly follows the calculation of the heritability, which can be a first approach to study the genetic influence on immune parameters in livestock. Heritability, or h^2, is a determination of how large a part of the measured response is heritable. Variation can be considered something undesirable reducing the significance of differences between experimental groups. At the same time however, variation, or phenotypic variance to animal breeders, is a highly interesting phenomenon that needs to be unravelled into additive and non-additive genetic variance on the one hand, and environmental variance on the other hand. As a consequence, the h^2 can be estimated as the ratio of the additive genetic variation and the phenotypic variation. Today, for a large number of immune parameters, estimations of the extent to which the individual its phenotype is determined by genes transmitted from the parents (h^2), have been calculated, comprising all important livestock species. Although it is not the aim of this paragraph to review these heritability estimates, one recent example should be noted. The number of polymorphonuclear leucocytes in the pig has been demonstrated to have such a high heritability (h^2=0.87) that single gene control was suspected (Edfors-Lilja *et al.*, 1994). The authors suggested this immune trait to be incorporated into future selection indices for the improvement of overall resistance.

The general outcome of the studies in both livestock and laboratory animals is that immune responsiveness and disease resistance are quantitative traits regulated by the effects of several genes that are influenced again by a variety of environmental factors. The selection of lines with high or low immune responsiveness, such as the Biozzi mouse lines, without prior selection for Mhc alleles, has pointed out that the Mhc is only one set of genes among many (Skamene and Pietrangeli, 1991), although with a central role in the generation of acquired immune responsiveness, and relatively well described. In general, breeding for resistance to specific diseases seems to be associated with breeding for specific immune responsiveness and most likely involves selection for certain Mhc haplotypes (Outteridge, 1993).

§ 1.5 Genetic regulation of disease resistance in fish

§ 1.5.1 Direct breeding for disease resistance

Fish diseases can be caused by a wide range of pathogenic organisms, and are a threat to intensive aquaculture. They can be controlled by management improvement, use of antibiotics or by selection for increased disease resistance. The latter approach especially, may constitute a long-term improvement of resistance to diseases. In fish, and generally true for all livestock species, the ability to resist infection is rarely manifested as an all-or none phenomenon. For that reason, dealing with infections, resistant and susceptible terminology is always comparative rather than absolute. In most cases, all animals are susceptible to the initial infection, but differ in their ability to limit the infection or destroy the pathogen. The best criterion for determining the level of resistance is survival, which reflects the cumulative effects of all host responses against prior stages in the life history of a parasite (Wassom and Kelly, 1990). Under farming conditions however, survival of fish is a very difficult trait to record and under influence of many environmental factors (Fjalestad *et al.*, 1993).

The history of direct breeding for disease resistant fish (brook trout; *Salvelinus fontinalis*) goes back as far as 1925, to Embody and Hayford who used the survivors after a natural outbreak of disease (probably furunculosis) to breed new generations with increased disease resistance. Since then, genetic variation in resistance to disease has frequently been established, and heritabilities for disease resistance have been estimated for a large number of fish species and diseases (for a review see Chevassus and Dorson, 1990). Direct breeding for survival of *Aeromonas salmonicida* infection in brook trout (*S. fontinalis*) and brown trout (*Salmo trutta*) by Wolf (1953) led to strains resistant to furunculosis, and was only recently ascribed to higher levels of mucus precipitin activity (Cipriano *et al.*, 1994). Probably the best known direct breeding for resistance to disease, however, was done in carp (*Cyprinus carpio* L.) by Kirpichnikov *et al.* for resistance to "infectious dropsy" (reviewed in 1993), which is now assumed to be caused by a combination of *Rhabdovirus carpio* and atypical *Aeromonas salmonicida* (Bootsma *et al.*, 1977). Healthy fish from one of three strains (Local, Ropsha and Ukranian x Ropsha) were selected for resistance to dropsy, and for increased growth rate. Although the comparative resistance of the Ropsha strain differed depending on the generation of selection, a general increase in resistance to infection with *R. carpio* could be demonstrated. In addition, in an experiment where Ukranian x Ropsha carp after five generations of selection were compared with fish of the previous three generations, an increased resistance to infectious dropsy with selection was shown.

§ 1.5.2 Experimental challenges

Challenge tests with infectious organisms are commonly used to establish genetic differences in disease resistance. A reproducible challenge methodology is very important for establishing genetic differences between families. Experimental challenge with atypical *A. salmonicida*, the causative agent of a carp skin disease called erythrodermatitis (Bootsma *et al.*, 1977), demonstrated genetic variation in resistance between carp strains of different geographical origin (van Muiswinkel *et al.*, 1990). This genetic variation in resistance could not be correlated to polymorphic differences in iron-chelating transferrin genotypes (Houghton *et al.*, 1991). A significant effect of individual females was observed, however. Challenge of the progeny of individual females taken from the above-mentioned carp strains, identified female parents with genetically determined differences in resistance to *A. salmonicida* (Wiegertjes *et al.*, 1993a). These differences in resistance to erythrodermatitis were shown to correlate to differences in survival under farming conditions (Wiegertjes *et al.*, 1995).

The importance of a controlled challenge methodology has been shown clearly by experimental manipulations of the challenge technique that identified two rainbow trout (*Oncorhynchus mykiss*) strains with differential susceptibility to infection with a myxosporean parasite; *Ceratomyxa shasta* (Ibarra *et al.*, 1991). The complete life cycle of this parasite is unknown, and experimental infections are achieved by placing fish in waters known to contain the infective stage. Both resistant (from the Pit River) and susceptible strains (from Mt. Shasta) succumbed to infections induced by the greatest concentration of parasites administered, but at the lowest dose examined only 13% of the resistant compared to 90% of the susceptible strain died over a 53 day period. Further study on the duration of the challenge exposure indicated a differential effect on the degree of dominance in the F1; an apparent dominance for susceptibility was found after continuous exposure, but a "pulse" exposure pointed at dominance for resistance (Ibarra *et al.*, 1992). A more extensive genetic analysis of susceptibility to *C. shasta* of F_1, F_2 and backcrosses to both strains, showed that no simple Mendelian model of inheritance fit the observed mortality data. No segregation in the distributions of time to death was detectable (Ibarra *et al.*, 1994). In fact, the phenotypic variance in the F_2 was lower than in the F_1. However, the Pit River alleles (low susceptibility) were dominant over the alleles of susceptibility (Mt. Shasta), although again affected by time of exposure to the parasite. The existence of two interacting mechanisms was postulated, one controlling the level of parasites by influencing invasion and/or establishment, the other involving the ability of the fish to mount an effective immune response against the parasites. Although the authors refer to the possibility that a lack of antibody formation could cause susceptibility, no data are available on the immune responsiveness of either strain.

§ 1.5.3 Genetic differences in disease resistance

The genetics of disease resistance in fish have been extensively reviewed by Chevassus and Dorson (1990). The present paragraph updates this review with some recent studies on this subject. Genetic differences in resistance to *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, have been established between a.o. coho salmon (*Oncorhynchus kisutch*) strains in British Columbia (Canada) with a different geographical origin (Withler and Evelyn, 1990). The most susceptible salmon (from the Robertson Creek) differed from the most resistant salmon (Kitimat River) in terms of survival and time to death, although large differences were observed between individual parents from the same strain. Comparison of the coho salmon strains from the Robertson Creek, characterized as comparatively susceptible, showed that some progenies expressed additional genetic variation in resistance to other bacteria, among which *Vibrio anguillarum* and *A. salmonicida* (Beacham and Evelyn, 1992). Again, significant effects of individual females were observed. An effort was made to explain the relative resistance of coho salmon from the Kitimat river, compared to coho salmon from the east coast of Vancouver Island, by examination of correlations with differences in non-specific immune mechanisms (Balfry, 1994; personal communication). Head kidney phagocytes from the resistant strain showed a significantly higher increase in respiratory burst activity after challenge with *V. anguillarum*, than phagocytes of the more susceptible strain, as compared to non-infected control fish (Balfry *et al.*, 1994).

The major importance of Atlantic salmon (*Salmo salar*) culture in Norway has led to an increasing number of reports on genetic differences in disease resistance. Gjedrem *et al.* (1991) reported a heritability (h^2=0.48) for resistance to furunculosis of Atlantic salmon families, after experimental challenge by co-habitation. Later, a genetic correlation was found between survival after experimental challenge (infection with a.o. *A. salmonicida*, *V. salmonicida*, or *R. salmoninarum*) and survival of Atlantic salmon under farming conditions, (Gjøen *et al.*, 1994). These results support the use of challenge tests as selective criteria for survival under farming conditions.

Another approach to study disease resistance of salmonid fish was taken by Fevolden *et al.* (1991). Atlantic salmon and rainbow trout were divergently selected for cortisol stress response. In the Atlantic salmon line selected for high cortisol levels, mortality under farming conditions, although apparently not caused by pathogens, was increased. Also, for the rainbow trout, the high-stress line was more susceptible to *A. salmonicida* but less susceptible to *V. anguillarum* challenge (Fevolden *et al.*, 1992). Serum levels of lysozyme, an enzyme with bactericidal activity, were higher in the high-stress rainbow trout line (Fevolden and Røed, 1993), which would support the use of lysozyme level as indirect selection criterion.

§ 1.5.4 Indirect selection: immune parameters

In fish, comparable with other farmed livestock species, data can be analyzed for associations with known (immuno)genetic markers, to demonstrate correlation with inherent differences in disease resistance. However, the value of (immunological) parameters to serve as markers for incorporation in a breeding program is highly dependent on their heritabilities and genetic correlation with survival (Fjalestad *et al.*, 1993). Indirect selection for disease resistance based upon immune parameters, often non-specific, has received increased attention. For example, the use of lysozyme level as indirect selection criterion was substantiated by a thoroughly established genetic variation in lysozyme activity in the rainbow trout (Røed *et al.*, 1993a; see Table 2).

Another, non-specific, defense mechanism that has been claimed responsible for, or linked to, differences in disease resistance between fish strains, is the complement system. Genetic variation in complement haemolytic activity has been reported for several fish species (Table 2), including the rainbow trout (Røed *et al.*, 1990), Atlantic salmon (Røed *et al.*, 1992, 1993a) and carp (Wiegertjes *et al.*, 1993b). However, genetic correlation to disease resistance has proven more difficult to demonstrate. Bactericidal activity in serum, although not clearly correlated with complement spontaneous haemolytic activity, has been positively correlated with heritable resistance to *A. salmonicida* in the rainbow trout (Hollebecq and Michel, 1989; Hollebecq *et al.*, 1991). Another approach was taken by Slierendrecht *et al.* (1993), where three different genotypes of rainbow trout C3, the central component of the complement system, were detected based on charge differences visualized in agarose gels (Jensen and Koch, 1991). These alloforms were observed to undergo a change in relative frequency in rainbow trout populations infected with viral haemorrhagic septicaemia (VHS) virus, and it was concluded that one of the genotypes ("f2") correlated with a decreased resistance to VHS.

An increasing number of publications report on the use of specific antibody levels to pathogenic organisms, in order to establish correlations with disease resistance of Atlantic salmon. Variation in antibody production against both *A. salmonicida* A-layer (Strønsheim *et al.*, 1994a) and *V. anguillarum* O-antigen (Strønsheim *et al.*, 1994b) has been shown to be heritable (see Table 2 for h^2 estimates). Similar to the analysis of genetic variation in lysozyme activity and spontaneous haemolytic activity (Røed *et al.*, 1993b), 34 full-sib families within 12 paternal half-sib groups, comprising a total of approximately 800 individually tagged fish, were analyzed. Surprisingly, the likelihood of surviving an experimental challenge with *V. anguillarum* was more affected by antibody titres against *A. salmonicida* than against *V. anguillarum* (Strønsheim *et al.*, 1994b). The same family material was used to study the genetic variation in the level of specific antibody production to pathogen-unrelated diphtheria

toxoid (DT; Eide *et al.*, 1994), and in serum iron levels, important for so-called nutritional immunity (Ravndal *et al.*, 1994). In contrast to the response against *A. salmonicida* or *V. anguillarum*, the Atlantic salmon had high pre-immune titres against DT, and generally showed a low antibody production to DT after immunization. In addition, the heritability estimate for antibody levels against DT in Atlantic salmon were relatively low (Table 2), reducing the value of this parameter for breeding purposes. An attempt to correlate a number of the immunological parameters to survival after challenge with *V. anguillarum* failed to produce a clear picture. It was concluded that the use of immunological traits such as described above, explained a too small amount of variation in survival to substantially aid selection for disease resistance (Zarnecki *et al.*, 1994).

Another series of experiments on the same family material was initiated after Gjedrem *et al.* (1991) reported a very high heritability (h^2=0.48) for resistance to furunculosis in Atlantic salmon families. To detect a potential immune response marker for resistance to furunculosis, the same 30 full-sib groups (unchallenged siblings) were analyzed for antibody production to *A. salmonicida* A-layer, or LPS, and for total level of immunoglobulin after immunization (Lund *et al.*, 1995a). Although the immunological significance of anti-*A. salmonicida* A-layer antibody production in the defense against furunculosis has not been established, it was shown that levels of these specific antibodies correlated positively with survival rates after experimental infection. The genetic variation in susceptibility of Atlantic salmon to furunculosis was later confirmed, and extended, with heritability estimates for susceptibility to *R. salmoninarum* and *V. salmonicida* in a study of 81 full-sib families within 32 sire progeny groups (Gjedrem and Gjoen, 1995). Interestingly, the estimated genetic correlations between susceptibility to the diseases involved, were all positive. Most of these full-sib families were subsequently examined for genetic variation in immune parameters such as the ones described above (Lund *et al.*, 1995b). In general, the heritability estimates were low (Table 2), and it was concluded that none of the parameters other than lysozyme showed any promise as a marker trait for selection to improve disease resistance.

Table 2. Heritability estimates for immune response parameters in fish. An overview.

Immune resp	Parameter	Assay	Fish species	h^2	Methodology	Reference
non-specific	SHA	plate assay	*O. mykiss*	0.13-0.41	family analysis	Røed *et al.* 1990
		,,	*S. salar*	0.32	,,	Røed *et al.* 1992
		,,	,,	0.04-0.23	,,	Røed *et al.* 1993a
		photometric	*C. carpio*	0.72 (H^2)	repeatability	Wiegertjes *et al.* 1993b
	THA	plate assay	*O. mykiss*	0.34-0.96	family analysis	Røed *et al.* 1990
		,,	*S. salar*	0.35	,,	Røed *et al.* 1992
		photometric	*C. carpio*	0.71 (H^2)	repeatability	Wiegertjes *et al.* 1993b
	C3 conc	immuno-electr	,,	0.54 (H^2)	,,	,,
	lysozyme	lysoplate assay	*O. mykiss*	0.22-0.27	family analysis	Røed *et al.* 1993b
	,,	,,	*S. salar*	0.02-0.20	,,	Røed *et al.* 1993a, Lund *et al.* 1995b
	total IgM	turbidimetric	,,	0.12	,,	Strønsheim *et al.* 1994b
	,,	ELISA	,,	0.00-0.40	,,	Lund *et al.* 1995b
specific	antibody	ELISA (*V. salm*)	*O. mykiss*	0.03-0.12	,,	,,
		,, (DT)	,,	0.20-0.23	,,	Eide *et al.* 1994
		,, (DT)	*S. salar*	0.06-0.12	,,	,,
		,, (*A. salm*)	,,	0.16-0.38	,,	Strønsheim *et al.* 1994a, Lund *et al.* 1995b
		,, (*V. ang*)	,,	0.18	,,	Strønsheim *et al.* 1994b
		,, (*V. salm*)	,,	0.19	,,	Lund *et al.* 1995b

h^2 = narrow heritability = measure for the hypothetical response to selection, H^2 = broad heritability = proportion of the total variance that is due to genetic factors, SHA = spontaneous and THA = total haemolytic activity, C3 = complement component C3, DT = diphtheria toxoid.

§ 1.5.5 Inbred fish lines

Indirect selection, especially marker selection based on immune parameters, remains an attractive approach to disease resistance of fish. Although sequence data of a number of expressed Mhc genes is available (reviewed by Dixon *et al.*, 1995), no association studies of the Mhc with disease resistance have been reported to date. More fundamental research on the genetic control of specific immune responsiveness is needed, before immune parameters can be exploited as indirect markers in selection schemes. The development of isogenic fish lines, or cloned lines, may add substantially to a thorough analysis of the relationship(s) between immune parameters and disease resistance. Fish can provide excellent experimental models for studies in embryology, neurobiology, endocrinology and environmental biology (Powers, 1989). In fact, because the immune defense mechanisms of fish are similarly competent as those of mammals, there is an increasing interest in the immune responses of fish as models for higher vertebrates in immunological/immunotoxicological studies (Enane *et al.*, 1993).

Some fish species produce cloned lines naturally. The first to be recognized was a live-bearing fish, *Poecilia formosa*, also named the Amazon molly, belonging to a group of about 50 known "species" of so-called unisexual vertebrates. Typically, they are almost exclusively female, of interspecific hybrid origin and reproduce clonally. The method of reproduction is called gynogenesis; eggs are produced without genetic recombination or without a reduction in ploidy. Subsequently, the eggs require sperm only to start embryonic development, and because syngamy of egg and sperm does not occur, the offspring are genetically identical (Dawley, 1989). Identical DNA fingerprints of *P. formosa* demonstrated that this species was clonally stable for at least three generations (Turner *et al.*, 1990). Recently, it was shown that small amounts of DNA from the sperm of the bisexual partner can sometimes incorporate, via microchromosomes, into the genome of *P. formosa* (Schartl *et al.*, 1995), however.

In general, unisexual vertebrates form a rather unexploited potential as models in cellular and molecular biology (Dawley, 1989), with the possible exception of the Japanese 'ginbuna' crucian carp (*Carassius gibelio langsdorfii*). Naturally occurring unisexual triploid populations of this particular fish species have been used as a model to study cellular immunology (Nakanishi, 1987a,b), and hybrid crosses with goldfish (*Carassius auratus*) have been used for histocompatibility-restricted scale transplantation experiments (Nakanishi, 1987c). The transfer of immunity by the intravascular introduction of splenic lymphoid cells, preferably taken at seven days after immunization, into naive recipients, was only successful in histocompatible donor-recipient systems (Nakanishi, 1987a, b). Although this is indeed one of the best models to study cellular immunology in fish, the ginbuna are heterozygous triploid animals and difficult to reproduce in the laboratory, hampering the use of this particular fish model.

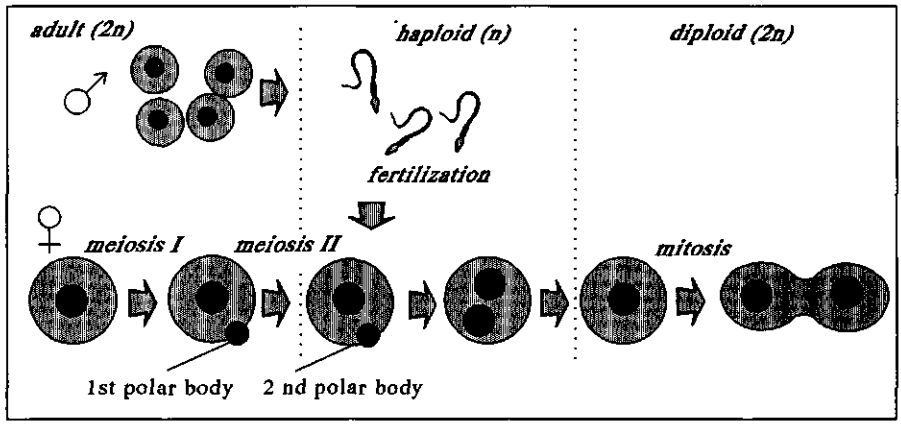
The possibilities for the laboratory production of defined fish lines are a few. In theory, similar to the inbreeding done in laboratory animals such as the mouse, mating of close relatives will lead to inbred lines after 20-30 generations (Falconer, 1989). In practice, this methodology is limited to fish species with a relatively short generation interval. Inbred lines have been developed by brother-sister mating in a number of teleosts, which have been used, for example, to estimate the number of histocompatibility loci (*Xiphophorus maculatus*: Kallman, 1964; *Oryzias latipes*; Hyodo-Taguchi and Egami, 1985; Matsuzaki and Shima, 1989), but in general these fish species are too small for immunobiological use.

Induced, or artificial, gynogenesis is essentially the same as some of the natural forms of gynogenesis, with the difference that induced gynogenesis can be readily controlled in the laboratory. Fertilization with sperm that is genetically inactivated by irradiation (often U.V. light) reduces the contribution of the male to a mere activation of the female egg (Fig. 4). The now haploid 'embryo' is treated with a shock (often temperature) at the moment of its first mitotic division, which causes the two haploid cells to fuse to one diploid cell (Fig. 4e). The embryo contains a duplicated set of maternal chromosomes and is therefore homozygous. Subsequent gynogenetic reproduction of such homozygous individuals will yield homozygous cloned lines. This gynogenetic reproduction is like the first one, except for the timing of the temperature shock, which is now at the moment of extrusion of the 2nd polar body (Fig. 4d).

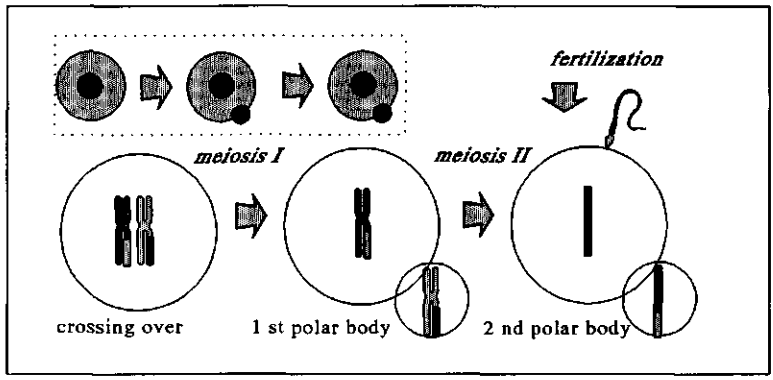
The first report on inbred fish lines produced by artificial gynogenesis was by Streisinger *et al.* (1981) using zebrafish (*Brachydanio rerio*). Since then, gynogenetic homozygous progeny has been produced for a large number of fish species, using a vast array of ploidy manipulation techniques (reviewed by Ihssen *et al.*, 1990), but apparent technical problems limited the production of inbred lines by artificial gynogenesis to three fish species beside the zebra fish; the medaka (*Oryzias latipes*: Naruse *et al.*, 1985), the ayu (*Plecoglossus altivelis*: Han *et al.*, 1991) and the common carp (*Cyprinus carpio*: Komen *et al.*, 1991). The large size that common carp can reach under laboratory circumstances makes this fish species particularly suitable for studies on cellular immunology.

Right page: **Figure 4a. Sexual reproduction**. The only haploid (*n*) cells are the products of meiosis. Meiosis in the oocyte is not completed until after fertilization by a sperm cell, which provides the male pronucleus. Male and female pronuclei fuse to form the diploid (*2n*) zygote. Replication of the chromosomes occurs prior to the first cleavage. **b. Details of meiosis**. For simplicity, only one chromosome pair is shown. DNA replication occurs before meiosis. The *2n* chromosomes now comprise four copies of the genome, which segregate through the two meitoic divisions. The haploid female pronucleus is the only surviving product; the other three copies are discarded in the 1st and 2nd polar bodies. As a result of crossing-over during meiosis I, each of the four copies can receive a different combination of maternal and paternal segments. **c. Details of mitosis**. Fusage of male and female pronuclei results in a diploid zygote which divides by mitosis, separating identical chromatids.

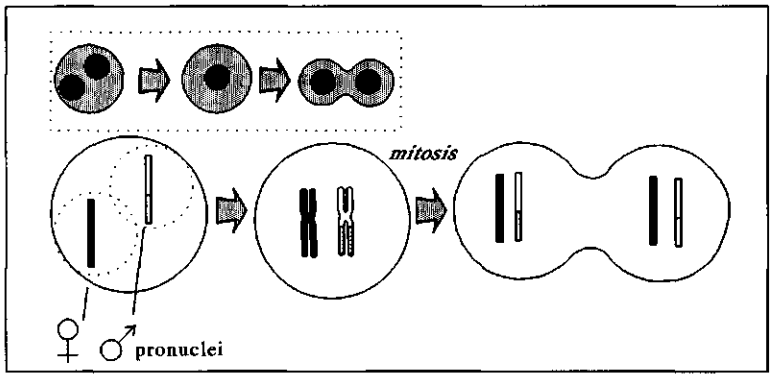
a


adult (2n)
haploid (n)
diploid (2n)
fertilization
meiosis I
meiosis II
mitosis
1st polar body
2 nd polar body

b


fertilization
meiosis I
meiosis II
crossing over
1 st polar body
2 nd polar body

c


mitosis
pronuclei

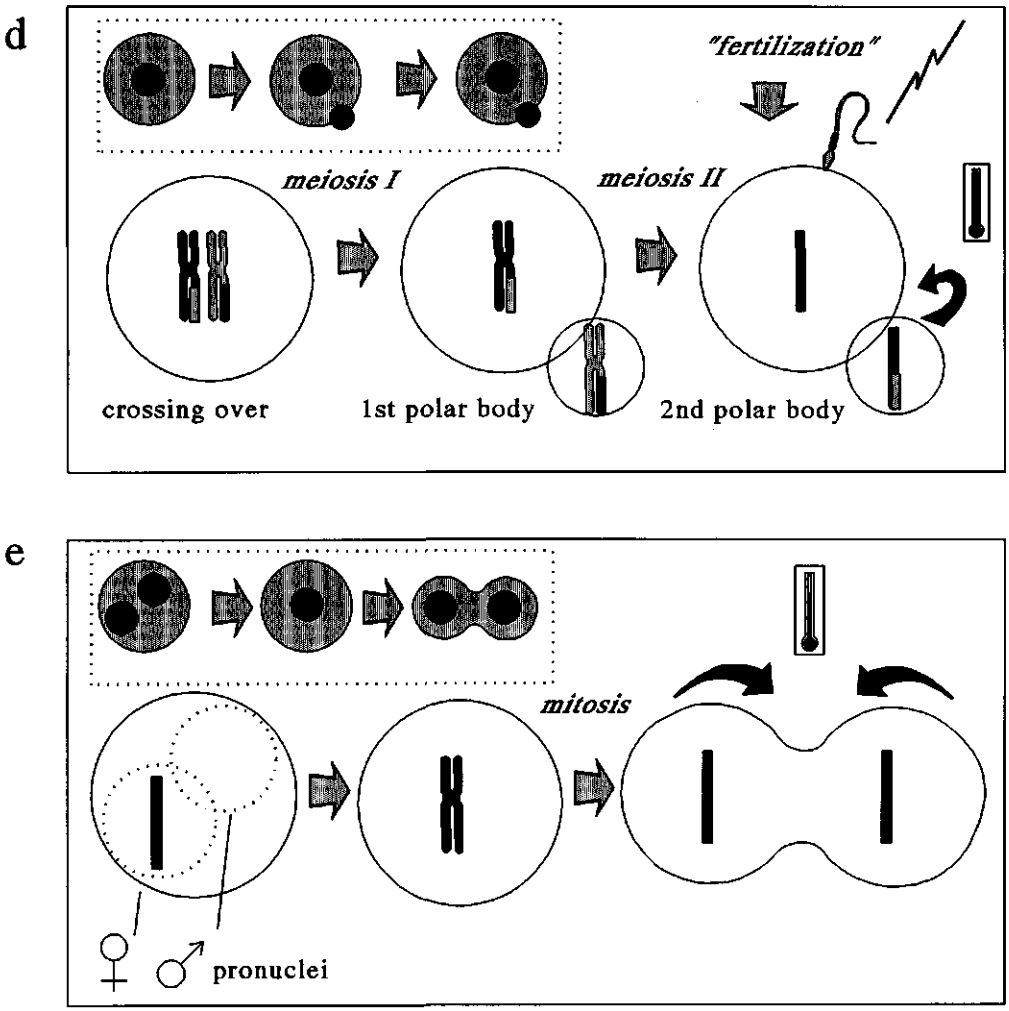


Figure 4d. Induced gynogenesis: **retention of the second polar body**. Activation of the oocyte with an irradiated sperm cell results in a haploid embryo. Cold shock treatment immediately after activation prevents the extrusion of the second polar body and will result in a diploid embryo (meiotic gynogenesis). Because of crossing-over events during meiosis, the gynogenetic embryo may be heterozygous, depending on the degree of homozygosity of the female parent. **e. Inhibition of the first mitotic division**. After activation of the oocyte with irradiated sperm, completion of meiosis is allowed. Heat shock treatment at the moment of first mitotic division prevents the haploid 'zygote' to divide, fusing the two cells, containing identical chromatids, into one diploid zygote.

The need for uniformity has often been the driving force behind the development of inbred lines of laboratory animals. For some purposes, it is indeed genetic uniformity that is required and, in general, evidence has accumulated that inbred (mouse) lines fully satisfy this requirement. For other purposes, however, it is phenotypic uniformity that is desired; the less variable the animals, the smaller the number that need to be used to attain a given degree of accuracy in measuring their response to a treatment. The value of inbred lines depends on how much of the variance can be removed by inbreeding, and on how much the environmental variance will be affected by inbreeding. In general, similar to findings in laboratory mice, an increase in the variation in quantitative traits, such as length and body weight, can be observed in homozygous families obtained through inhibition of the first mitotic division (*P. altivelis*, Taniguchi *et al.*, 1990, 1994; *C. carpio*, Sumantadinata *et al.*, 1990, Komen *et al.*, 1992). The homozygous genotype can suffer developmental instability, which may cause additional environmental variance, and may sometimes offset the reduced genetic variance, so that phenotypically an inbred line can be even more variable than a non-inbred strain. As has been shown for the quantitative variation in body length and weight in gynogenetic carp (Komen *et al.*, 1993), the way to obtain genetic uniformity without increased environmental variation is to use the F_1 of a cross between two homozygous animals. The effects of the generally low hatching rates observed with induced gynogenesis, which suggest the existence of a strong natural selection against deleterious genes, are overcome by the large fecundity of fish species such as the common carp.

Rationale and outline of this thesis

Studies in both livestock and laboratory animals indicate that immune responsiveness and disease resistance can be considered quantitative traits regulated by several genes, under influence of a variety of environmental factors. The selection of lines with high or low immune responsiveness, such as initiated by Biozzi *et al.* for the mouse, has pointed out that the Mhc is only one set out of the multitude of genes involved in the ability of the host to resist infection. In general, however, breeding for resistance to specific diseases has invariably resulted in a selection for certain Mhc haplotypes. The strategy that can be taken to study genetic regulation of disease resistance is highly dependent upon the availability of inbred lines of the animal species under investigation. In fish, such as the Atlantic salmon, immune parameters, often non-specific, have been examined for their use in indirect breeding schemes. However, attempts to correlate genetic differences in immune responsiveness with survival after experimental challenge with pathogenic bacteria, have failed to define immune parameters that can substantially aid selection for genetic disease resistance. The development of homozygous cloned carp lines, via gynogenetic reproduction, or hybrid but isogenic lines, will

add to a thorough analysis of the immunogenetic relationship(s) between immune responsiveness and disease resistance in fish. The aim of the work described in this thesis is to investigate the possibility to pre-select a particular immune parameter in order to produce isogenic carp lines, via gynogenesis, that express the trait under selection. If possible, this would allow the repeated production of numerous isogenic lines, all selected for different immunological parameters. As a model, we chose a defined antigenic determinant (dinitrophenyl) in combination with a reliable read-out system (enzyme-linked immunosorbent assay), to divergently select carp for their primary antibody response. The use of simple hapten antigenic determinants may increase the detection level of genetic control of the immune response, and can facilitate subsequent analysis of inherent differences.

A base population of carp, consisting of a single hybrid cross, was immunized with a hapten-carrier complex, dinitrophenyl-keyholelimpet haemocyanin (DNP-KLH). Subsequently, animals were divergently selected for their antibody response to DNP. Three individuals defined as high and three defined as low responding, were gynogenetically reproduced to obtain corresponding homozygous families within one generation. Upon immunization with DNP-KLH, the antibody response was found to be significantly higher in the high-responder offspring (**chapter 2**). Based upon these results, the selection procedure was continued with two of these families, one high-responder and one low-responder (**chapter 3**). Simultaneously, a parasite infection model was developed, for which susceptibility to the haemoflagellate *Trypanoplasma borreli* was shown to be under genetic influence, possibly due to a reduced humoral responsiveness of susceptible carp lines (**chapter 4**). Also, one high-responder and one low-responder family were used to examine the immune responsiveness to hapten-carrier complexes other than DNP-KLH, and to investigate the resistance to infection with the haemoflagellate parasite (**chapter 5**). Subsequently, a number of selected animals, both homozygous females and functional males, were crossed to produce several isogenic F_1 hybrid lines with presumed high or low responsiveness to DNP-KLH. The clonal nature of these lines was confirmed with DNA fingerprinting, and, similar to previous experiments, the ubiquitous nature of the differences in immune responsiveness was examined by immunization with several hapten-carrier complexes, and by infection with *T. borreli* (**chapter 6**). An effort was made to explain the selected differences in immune responsiveness, by examination of a possible association with Mhc class II polymorphism (**chapter 6**). The possibilities implicated by the selection strategy are outlined and discussed in **chapter 7**.

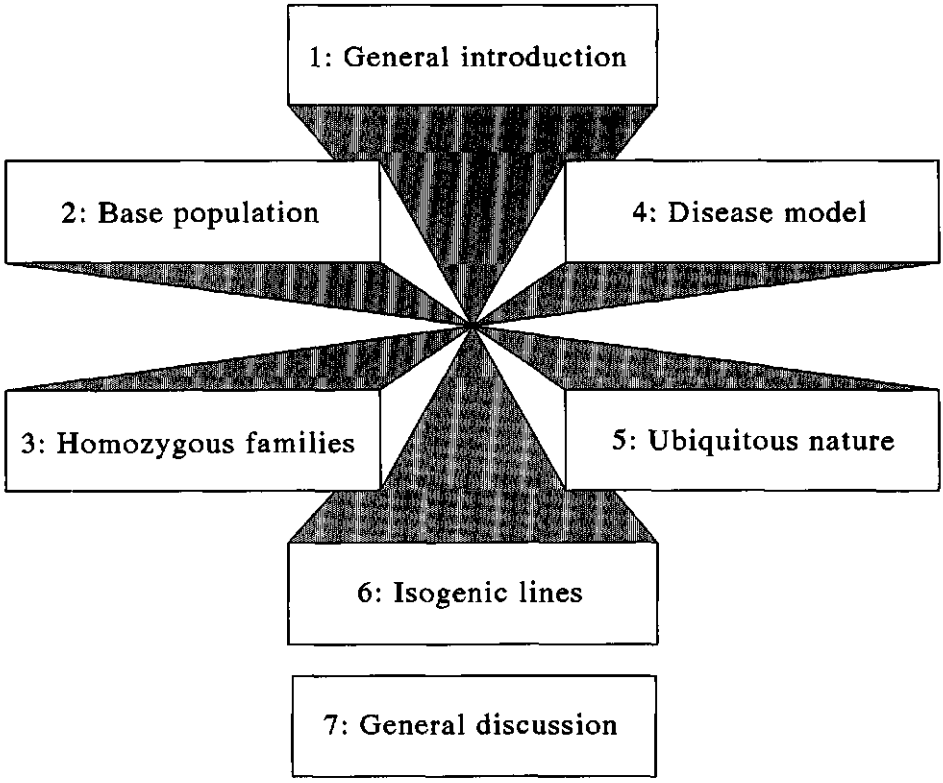


Figure 5. Schematic overview of this thesis's structure.

Acknowledgements

We thank Drs. Hans Komen and Jim Daly for critical reading of the manuscript and useful comments.

References

Andersson, L. 1994. The role of Mhc polymorphism in disease/parasite resistance. In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph, Vol. XXI, pp. 177-182.

Ash, C. 1991. Macrophages at the centre of infection. Parasitol. Today 7, 2-3.

Bacon, L.D. 1992. Measurement of immune competence in chickens. Poultry Sci. Rev. 4, 187-195.

Bacon, L.D. and Witter, R.L. 1992. Influence of Turkey herpesvirus vaccination on the *B*-haplotype effect on Marek's disease resistance in 15.B-congenic chickens. Avian Dis. 36, 378-385.

Balfry, S.K., Iwama, G.K. and Evelyn, T.P.T. 1994. Components of the non-specific immune system in Coho salmon associated with strain differences in innate disease resistance. Dev. Comp. Immunol. 18, S82.

Beacham, T.D. and Evelyn, T.P.T. 1992. Genetic variation in disease resistance and growth of Chinook, Coho, and Chum salmon with respect to vibriosis, furunculosis, and bacterial kidney disease. Trans. Am. Fish. Soc. 121, 456-485.

Biozzi, G., Mouton, D., Sant'Anna, O.A., Passos, H.C., Gennari, M., Reis, M.H., Ferreira, V.C.A., Heumann, A.M., Bouthillier, Y., Ibanez, O.M., Stiffel, C. and Siqueira, M. 1979. Genetics of immunoresponsiveness to natural antigens in the mouse. Curr. Top. Microbiol. Immunol. 85, 31-98.

Biozzi, G., Mouton, D., Stiffel, C. and Bouthillier, Y. 1984. A major role of the macrophage in quantitative genetic regulation of immunoresponsiveness and antiinfectious immunity. Advances in Immunol. 36, 189-233.

Blackwell, J.M. 1988a. Bacterial infections. In: Genetics of resistance to bacterial and parasitic infection (eds: Wakelin, D.M. and Blackwell, J.M.). Taylor & Francis, New York, pp. 63-101.

Blackwell, J.M. 1988b. Protozoan infections. In: Genetics of resistance to bacterial and parasitic infection (eds: Wakelin, D.M. and Blackwell, J.M.). Taylor & Francis, New York, pp. 103-151.

Blackwell, J.M., Roach, T.I.A., Atkinson, S.E., Ajioka, J.W., Barton, C.H. and Shaw, M-A. 1991. Genetic regulation of macrophage priming/activation: the *Lsh* story. Immunol. Lett. 30, 241-248.

Bootsma, R., Fijan, N. and Blommaert, J. 1977. Isolation and preliminary identification of the causative agent of carp erythrodermatitis. Veterinarski Archiv, Zagreb 47, 291-302.

Briles, W.E., Stone, H.A. and Cole, R.K. 1977. Marek's disease: Effects of *B* histocompatibility alloalleles in resistant and susceptible chicken lines. Science 195, 193-195.

Buschmann, H., Kräusslich, H., Herrmann, H., Meyer, J. and Kleinschmidt, A. 1985. Quantitative immunological parameters in pigs - experiences with the evaluation of an immunocompetence profile. Z. Tierz. Züchtungsbiol. 102, 189-199.

Chevassus, B and Dorson, M. 1990. Genetics of resistance to disease in fishes. Aquaculture 85, 83-107.

Cipriano, R.C., Ford, L.A. and Jones, T.E. 1994. Relationship between resistance of salmonids to furunculosis and recovery of *Aeromonas salmonicida* from external mucus. J. Wildl. Dis. 30, 577-580.

Colombani, M.J., Pla, M., Mouton, D. and Degos, L. 1979. *H-2* Typing of mice genetically selected for high or low antibody production. Immunogenetics 8, 237-243.

Covelli, C., Mouton, D., Di Majo, C., Bouthillier, Y., Bangrazi, C., Mevel, J-C., Rebessi, S., Doria, G. and Biozzi, G. 1989. Inheritance of immune responsiveness, life span, and disease incidence in interline crosses of mice selected for high or low multispecific antibody production. J. Immunol. 142, 1224-1234.

Dawley, R.M. 1989. An introduction to unisexual vertebrates. In: Evolution and ecology of unisexual vertebrates (eds: Dawley, R.M. and Bogart, J.P.). New York State Museum, Albany, Bull. 466, pp. 1-18.

Dixon, B., van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. 1995. Fish major histocompatibility complex genes: an expansion. Dev. Comp. Immunol. 19, 109-133.

Doenhoff, M.J. and Davies, A.J.S. 1991. Genetic improvement of the immune system: possibilities for animals. In: Breeding for disease resistance in farm animals (eds: Owen, J.B. and Axford, R.F.E.). C.A.B. Int., Wallingford, UK, pp. 24-53.

Edfors-Lilja, I., Wattrang, E., Magnusson, U. and Fossum, C. 1994. Genetic variation in parameters reflecting immune competence of swine. Vet. Immunol. Immunopathol. 40, 1-16.

Eide, D.M., Linder, R.D., Strønsheim, A., Fjalestad, K.T., Larsen, H.J.S. and Røed, K.H. 1994. Genetic variation in antibody response to diphtheria toxoid in Atlantic salmon and rainbow trout. Aquaculture 127, 103-113.

Embody, G.C. and Hayford, C.O. 1925. The advantage of rearing brook trout fingerlings from selected breeders. Trans. Am. Fish. Soc. 55, 135-138.

Enane, N.A., Frenkel, K., O'Connor, J.M., Squibb, K.S. and Zelikoff, J.T. 1993. Biological markers of macrophage activation: applications for fish phagocytes. Immunology 80, 68-72.

Falconer, D.S. 1989. Introduction to quantitative genetics. John Wiley & Sons Inc., New York.

Festing, M.F.W. and Blackwell, J.M. 1988. Determination of mode of inheritance of host response. In: Genetics of resistance to bacterial and parasitic infection (eds: Wakelin, D.M. and Blackwell, J.M.). Taylor & Francis, New York, pp. 21-61.

Fevolden, S.E., Refstie, T. and Røed, K.H. 1991. Selection for high and low cortisol stress response in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Aquaculture 95, 53-65.

Fevolden, S.E., Refstie, T. and Røed, K.H. 1992. Disease resistance in rainbow trout (*Oncorhynchus mykiss*) selected for stress response. Aquaculture 104, 19-29.

Fevolden, S.E. and Røed, K.H. 1993. Cortisol and immune characteristics in rainbow trout (*Oncorhynchus mykiss*) selected for high or low tolerance to stress. J. Fish Biol. 43, 919-930.

Fjalestad, K.T., Gjedrem, T. and Gjerde, B. 1993. Genetic improvement of disease resistance in fish: an overview. Aquaculture 111, 65-74.

Frangoulis, B., Mouton, D., Sant'Anna, O.A., Vidard, L. and Pla, M. 1990. *H-2* typing of mice genetically selected for high or low antibody production. Immunogenetics 31, 389-392.

Gavora, J.S and Spencer, J.L. 1983. Breeding for immune responsiveness and disease resistance. Anim. Bl. Gr. Biochem. Genet. 14, 159-180.

Gjedrem, T. and Gjøen, H.M. 1995. Genetic variation in susceptibility of Atlantic salmon, *Salmo salar* L., to furunculosis, BKD and cold water vibriosis. Aquacult. Research 26, 129-134.

Gjedrem, T., Salte, R. and Gjøen, H.M. 1991. Genetic variation in susceptibility of Atlantic salmon to furunculosis. Aquaculture 97, 1-6.

Gjøen, H.M., Refstie, T., Gjedrem, T. and Gjerde, B. 1994. Genetic correlation between survival under farming conditions and challenge tests in Atlantic salmon. In: Proceedings of the 5th International Symposium on Genetics in Aquaculture, Halifax, p. 47.

Glass, E.J., Oliver, R.A., Collen, T., Doel, T.R., Dimarchi, R. and Spooner, R.L. 1991a. Mhc class II restricted recognition of FMDV peptides by bovine T cells. Immunology 74, 594-599.

Glass, E.J., Oliver, R.A. and Spooner, R.L. 1990. Variation in T cell responses to ovalbumin in cattle: evidence for Ir gene control. Anim. Genet. 21, 15-28.

Glass, E.J., Oliver, R.A. and Spooner, R.L. 1991b. Bovine T cells recognize antigen in association with Mhc class II haplotypes defined by one-dimensional isoelectric focusing. Immunology 72, 380-385.

Groves, T.C., Wilkie, B.N., Kennedy, B.W. and Mallard, B.A. 1993. Effect of selection of swine for high or low immune responsiveness on monocyte superoxide anion production and class II Mhc antigen expression. Vet. Immunol. Immunopathol. 36, 347-358.

Han, H-S., Taniguchi, N. and Tsujimura, A. 1991. Production of clonal ayu by chromosome manipulation and confirmation by isozyme marker and tissue grafting. Nippon Suisan Gakkaishi 57, 825-832.

Hollebecq, M-G. and Michel, C. 1989. Evidence for a maternal effect in furunculosis resistance of rainbow trout. In: Proceedings of the 4th Conference of the European Association of Fish Pathologists, Santiago de Compostela, p.51.

Hollebecq, M-G., Michel, C., Faivre, B. and Kerouault, B. 1991. Possibilities of selection for furunculosis (*Aeromonas salmonicida*) resistance in rainbow trout (*Oncorhynchus mykiss*). In: Proceedings of the 5th Conference of the European Association of Fish Pathologists, Budapest, p.163.

Houghton, G., Wiegertjes, G.F., Groeneveld, A. and van Muiswinkel, W.B. 1991. Differences in disease resistance of carp, *Cyprinus carpio* L., to atypical *Aeromonas salmonicida*. J. Fish Dis. 14, 333-341.

Hyodo-Taguchi, Y. and Egami, N. 1985. Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. Zool. Sci. 2, 305-316.

Ibarra, A.M., Gall, G.A.E. and Hedrick, R.P. 1991. Susceptibility of two strains of rainbow trout *Oncorhynchus mykiss* to experimentally induced infections with the myxosporean *Ceratomyxa shasta*. Dis. aquat. Org. 10, 191-194.

Ibarra, A.M., Hedrick, R.P. and Gall, G.A.E. 1992. Inheritance of susceptibility to *Ceratomyxa shasta* (Myxozoa) in rainbow trout and the effect of length of exposure on the liability to develop ceratomyxosis. Aquaculture 104, 217-229.

Ibarra, A.M., Hedrick, R.P. and Gall, G.A.E. 1994. Genetic analysis of rainbow trout susceptibility to the myxosporean, *Ceratomyxa shasta*. Aquaculture 120, 239-262.

Ihssen, P.E., McKay, L.R., McMillan, I. and Phillips, R.B. 1990. Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Trans. Am. Fish. Soc. 119, 698-717.

Jensen, B.L. and Koch, C. 1991. Genetic polymorphism of component C3 of rainbow trout (*Oncorhynchus mykiss*) complement. Fish Shellf. Immunol. 1, 237-242.

Kallman, K.D. 1964. An estimate of the number of histocompatibility loci in the teleost *Xiphophorus maculatus*. Genetics 50, 583-595.

Kaufman, J., Volk, H. and Wallny, H-J. 1995. A "minimal essential Mhc" and an "unrecognized Mhc": two extremes in selection for polymorphism. Immunol. Rev. 143, 63-88.

Kean, R.P., Briles, W.E., Cahaner, A., Freeman, A.E. and Lamont, S.J. 1994a. Differences in major histocompatibility complex frequencies after multitrait, divergent selection for immunocompetence. Poultry Sci. 73, 7-17.

Kean, R.P., Cahaner, A., Freeman, A.E. and Lamont, S.J. 1994b. Direct and correlated responses to multitrait, divergent selection for immunocompetence. Poultry Sci. 73, 18-32.

Kierszenbaum, F. and Howard, J.G. 1976. Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. J. Immunol. 116, 1208-1211.

Kierszenbaum, F. and Sztein, M.B. 1990. Mechanisms underlying immunosuppression induced by *Trypanosoma cruzi*. Parasitol. Today 6, 261-264.

Kirpichnikov, V.S., Ilyasov, Ju.I., Shart, L.A., Vikhman, A.A., Ganchenko, M.V., Ostashevsky, A.L., Simonov, V.M., Tokhonov, G.F. and Tjurin, V.V. 1993. Selection of Krasnodar common carp (*Cyprinus carpio* L.) for resistance to dropsy: principal results and prospects. Aquaculture 111, 7-20.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley & Sons, New York.

Koch, C. and Simonsen, M. 1977. Immune response genes in chickens. Antibody responses to TGAL and GT. Immunogenetics 5, 161-170.

Kofler, R., Geley, S., Kofler, H. and Helmberg, A. 1992. Mouse variable-region gene families: complexity, polymorphism and use in non-autoimmune responses. Immunol. Rev. 128, 5-21.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman, E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.). II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

Komen, J., Eding, E.H., Bongers, A.B.J. and Richter, C.J.J. 1993. Gynogenesis in common carp (*Cyprinus carpio* L.). IV. Growth, phenotypic variation and gonad differentiation in normal and methyltestosterone-treated homozygous clones and F1 hybrids. Aquaculture 111, 271-280.

Komen, J., Wiegertjes, G.F., van Ginneken, V.J.T., Eding, E.H. and Richter, C.J.J. 1992. Gynogenesis in common carp (*Cyprinus carpio* L.) III. The effects of inbreeding on gonadal development of heterozygous and homozygous gynogenetic offspring. Aquaculture 104, 51-66.

Kreukniet, M.B., Gianotten, N., Nieuwland, M.G.B. and Parmentier, H.K. 1994a. *In vitro* T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. Poultry Sci. 73, 336-340.

Kreukniet, M.B., van der Zijpp, A.J. and Nieuwland, M.G.B. 1994b. Effects of route of immunization, adjuvant and unrelated antigens on the humoral immune response in lines of chickens selected for antibody production against sheep erythrocytes. Vet. Immunol. Immunopathol. 33, 115-127.

Lacey, C., Wilkie, B.N., Kennedy, B.W. and Mallard, B.A. 1989. Genetic and other effects on bacterial phagocytosis and killing by cultured peripheral blood monocytes of *SLA*-defined miniature pigs. Anim. Genet. 20, 371-382.

Lewin, H.A. 1989. Disease resistance and immune response genes in cattle: strategies for their detection and evidence for their existence. J. Dairy Sci. 72, 1334-1348.

Lie, Ø., Solbu, H., Larsen, H.J. and Spooner, R.L. 1986. Possible association of antibody responses to human serum albumin and (T-G)-A--L with the bovine major histocompatibility complex (*BoLa*). Vet. Immunol. Immunopathol. 11, 333-350.

Liu, G.Y., Baker, D., Fairchild, S., Figueroa, F., Quartey-Papafio, R., Tone, M., Healy, D., Cooke, A., Turk, J.L. and Wraith, D.C. 1993. Complete characterization of the expressed immune response genes in Biozzi Ab/H mice: structural and functional identity between Ab/H and NOD *A* region molecules. Immunogenetics 37, 296-300.

Locksley, R.M. and Scott, P. 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. In: Immunoparasitology Today (eds: Ash, C. and Gallagher, R.B.). Elsevier Trends Journals, Cambridge, A58-A61.

Lund, T., Chiayvareesajja, J., Larsen, H.J.S. and Røed, K.H. 1995a. Antibody response after immunization as a potential marker for improved resistance against furunculosis. Fish Shellf. Immunol., in press.

Lund, T., Gjedrem, T., Bentsen, H.B., Eide, D.M., Larsen, H.J.S. and Røed, K.H. 1995b. Genetic variation in immune parameters and associations to survival in Atlantic salmon. J. Fish Biol. 46, 748-758.

Lundén, A., Sigurdardóttir, S., Edfors-Lilja, I., Danell, B., Rendel, J. and Andersson, L. 1990. The relationship between bovine major histocompatibility complex class II polymorphism and disease studied by use of bull breeding values. Anim. Genet. 21, 221-232.

Mallard, B.A., Wilkie, B.N. and Kennedy, B.W. 1989. Genetic and other effects on antibody and cell mediated immune response in swine leucocyte antigen (*SLA*) - defined miniature pigs. Anim. Genet. 20, 167-178.

Mallard, B.A., Wilkie, B.N., Kennedy, B.W. and Quinton, M. 1992. Use of estimated breeding values in a selection index to breed Yorkshire pigs for high or low immune and innate resistance factors. Anim. Biotechn. 3, 257-280.

Martin, A., Dunnington, E.A., Briles, W.E., Briles, R.W. and Siegel, P.B. 1989. Marek's disease and major histocompatibility complex haplotypes in chickens selected for high or low antibody response. Anim. Genet. 20, 407-414.

Martin, A., Dunnington, E.A., Gross, W.B., Briles, W.E., Briles, R.W. and Siegel, P.B. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody responses to sheep erythrocytes. Poultry Sci. 69, 871-878.

Matsuzaki T. and Shima A. 1989. Number of major histocompatibility loci in inbred strains of the fish *Oryzias latipes*. Immunogenetics 30, 226-228.

McDevitt, H.O. and Sela, M. 1965. Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. J. Exp. Med. 122, 517-531.

McDevitt, H.O. and Sela, M. 1967. Genetic control of the antibody response. II. Further analysis of the specificity of determinant-specific control, and genetic analysis of the response to (H-G)-A--L in CBA and C57 mice. J. Exp. Med. 126, 969-978.

Mejdell, C.M., Lie, Ø., Solbu, H., Arnet, E.F. and Spooner, R.L. 1994. Association of major histocompatibility complex antigens (*BoLa-A*) with AI bull progeny test results for mastitis, ketosis and fertility in Norwegian cattle. Anim. Genet. 25, 99-104.

Mosmann, T.R. and Moore, K.W. 1991. The role of Il-10 in crossregulation of T_H1 and T_H2 responses. In: Immunoparasitology Today (eds: Ash, C. and Gallagher, R.B.). Elsevier Trends Journals, Cambridge, A49-A.53.

Muiswinkel van, W.B., Komen, J., Pourreau, C.N., Houghton, G. and Wiegertjes, G.F. 1990. Disease resistance in different carp lines. In: Proceedings of the 4th World Congress on Genetics Applied to Livestock Production, Edinburgh, Vol. XVI, pp. 174-175.

Nakanishi, T. 1987a. Kinetics of transfer of immunity by immune leucocytes and PFC response to HRBC in isogeneic ginbuna crucian carp. J. Fish Biol. 30, 723-729.

Nakanishi, T. 1987b. Transferability of immune plasma and pronephric cells in isogeneic, allogeneic and xenogeneic transfer systems in crucian carp. Dev. Comp. Immunol. 11, 521-528.

Nakanishi, T. 1987c. Histocompatibility analyses in tetraploids induced from clonal triploid crucian carp and in gynogenetic diploid goldfish. J. Fish Biol. 31, 35-40.

Naruse, K., Ijiri, K., Shima, A. and Egami, N. 1985. The production of cloned fish in the medaka (*Oryzias latipes*). J. Exp. Zool. 236, 335-341.

Østergård, H., Kristensen, B. and Andersen, Ø. 1989. *Investigations in farm animals of associations* between Mhc system and disease resistance and fertility. Livest. Prod. Sci. 22, 49-67.

Outteridge, P.M. 1993. High and low responsiveness to vaccines in farm animals. Immunol. Cell Biol. 71, 355-366.

Parmentier, H.K., Siemonsma, R. and Nieuwland, M.G.B. 1994. Immune responses to bovine serum albumin in chicken lines divergently selected for antibody responses to sheep red blood cells. Poultry Sci. 73, 825-835.

Pinard, M.H., van Arendonk, J.A.M., Nieuwland, M.G.B. and van der Zijpp, A.J. 1992. Divergent selection for immune responsiveness in chickens. Estimation of realized heritability with an animal model. J. Anim. Sci. 70, 2986-2993.

Pinard, M.H., Janss, L.L.G., Maatman, R., Noordhuizen, J.P.T.M. and van der Zijpp, A.J. 1993. Effect of divergent selection for immune responsiveness and of major histocompatibility complex on resistance to Marek's disease in chickens. Poultry Sci. 72, 391-402.

Pinard, M.H. and van der Zijpp, A.J. 1993. Effect of major histocompatibility complex types in F1 and F2 crosses of chicken lines selected for immune responsiveness. Genet. Sel. Evol. 25, 191-203.

Powers, D.A. 1989. Fish as model systems. Science 246, 352-358.

Ravndal, J., Løvold, T., Bentsen, H.B., Røed, K.H., Gjedrem, T. and Rørvik, K-A. 1994. Serum iron levels in farmed Atlantic salmon: family variation and associations with disease resistance. Aquaculture 125, 37-45.

Røed, K.H., Brun, E., Larsen, H.J. and Refstie, T. 1990. The genetic influence on serum haemolytic activity in rainbow trout. Aquaculture 85, 109-117.

Røed, K.H., Fjalestad, K.T., Larsen, H.J. and Midthjel, L. 1992. Genetic variation in haemolytic activity in Atlantic salmon (*Salmo salar*). J. Fish Biol. 40, 739-750.

Røed, K.H., Fjalestad, K.T. and Strønsheim, A. 1993a. Genetic variation in lysozyme activity and spontaneous haemolytic activity in Atlantic salmon (*Salmo salar*). Aquaculture 114, 19-31.

Røed, K.H., Larsen, H.J.S., Linder, R.D. and Refstie, T. 1993b. Genetic variation in lysozyme activity in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 109, 237-244.

Rothschild, M.F. 1991. Selection under challenging environments. In: Breeding for disease resistance in farm animals (eds: Owen, J.B. and Axford, R.F.E). C.A.B. Int., Wallingford, UK, pp. 123-135.

Schartl, M., Nanda, I., Schlupp, I., Wilde, B., Epplen, J.T., Schmid, M. and Parzefall, J. 1995. Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish. Nature 373, 68-71.

Schat, K.A., Taylor, R.L. and Briles, W.E. 1994. Resistance to Marek's disease in chickens with recombinant haplotypes of the major histocompatibility (*B*) complex. Poultry Sci. 73, 502-508.

Schurr, E., Malo, D., Radzioch, D., Buschman, E., Morgan, K., Gros, P. and Skamene, E. 1991. Genetic control of innate resistance to mycobacterial infections. In: Immunoparasitology Today (eds: Ash, C. and Gallagher, R.B.). Elsevier Trends Journals, Cambridge. A42-A45.

Schwartz, R.H. 1986. Immune response (Ir) genes of the murine major histocompatibility complex. Advances in Immunol. 38, 31-201.

Skamene, E. and Pietrangeli, C.E. 1991. Genetics of the immune response to infectious pathogens. Curr. Opin. Immunol. 3, 511-517.

Slierendrecht, W.J., Jensen, L.B., Hørlyck, V. and Koch, C. 1993. Genetic polymorphism of complement component C3 in rainbow trout (*Oncorhynchus mykiss*) and resistance to viral haemorrhagic septicaemia. Fish Shellf. Immunol. 3, 199-206.

Stiffel, C., Liacopoulos-Briot, M., Decreusefond, C. and Lambert, F. 1977. Genetic selection of mice for quantitative responsiveness of lymphocytes to phytohemagglutinin. Eur. J. Immunol. 7, 291-297.

Stiffel, C., Liacopoulos-Briot, M., Decreusefond, C. and Lambert, F. 1983. *In vitro* viability of lymphoid cells from lines of mice genetically selected for high or low responsiveness to phytohemagglutinin. Cell. Immunol. 77, 77-91.

Stiffel, C., Liacopoulos-Briot, M., Decreusefond, C. and Parlebas, J. 1987. Genetic difference in the proliferative response to T mitogens between Hi/PHA and Lo/PHA lymphocytes is independent of accessory cell function. Exp. Clin. Immunogenet. 4, 37-47.

Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. 1981. Production of homozygous diploid zebra fish (*Brachydanio rerio*) Nature 291, 293-296.

Strønsheim, A., Eide, D.M., Fjalestad, K.T., Larsen, H.J.S. and Røed, K.H. 1994a. Genetic variation in the humoral immune response in Atlantic salmon (*Salmo salar*) against *Aeromonas salmonicida* A-layer. Vet. Immunol. Immunopathol. 41, 341-352.

Strønsheim, A., Eide, D.M., Hofgaard, P.O., Larsen, H.J.S., Refstie, T. and Røed, K.H. 1994b. Genetic variation in the humoral immune response against *Vibrio salmonicida* and in antibody titre against *Vibrio anguillarum* and total IgM in Atlantic salmon (*Salmo salar*). Vet. Immunol. Immunopathol. 44, 85-95.

Sumantadinata, K., Taniguchi, N. and Sugiarto. 1990. Increased variance of quantitative characters in the two types of gynogenetic diploids of Indonesian common carp. Nippon Suisan Gakkaishi 56, 1979-1986.

Taniguchi, N., Han, H.S. and Tsujimura, A. 1994. Variation in some quantitative traits of clones produced by chromosome manipulation in ayu, *Plecoglossus altivelis*. Aquaculture 120, 53-60.

Taniguchi, N., Hatanaka, H. and Seki, S. 1990. Genetic variation in quantitative characters of meiotic- and mitotic- gynogenetic diploid ayu, *Plecoglossus altivelis*. Aquaculture 85, 223-233.

Teale, A.J. 1994. Conventional and molecular immunogenetics: potential impact on livestock improvement. In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph, Vol. XX, pp. 215-221.

Turner, B.J., Elder Jr., J.F., Laughlin, F. and Davis, W.P. 1990. Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting. Proc. Natl. Acad. Sci. USA 87, 5653-5657.

Vaiman, M., Metzger, J-J., Renard, C. and Vila, J-P. 1978. Immune response gene(s) controlling the humoral anti-lysozyme response (Ir-Lys) linked to the major histocompatibility complex *SL-A* in the pig. Immunogenetics 7, 231-238.

Vidal, S.M., Malo, D., Vogan, K., Skamene, E. and Gros, P. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for *Bcg*. Cell 73, 469-485.

Wakelin, D.M. 1988. Introduction. In: Genetics of resistance to bacterial and parasitic infection (eds: Wakelin, D.M. and Blackwell, J.M.). Taylor & Francis, New York, pp. 1-20.

Warner, C.M., Meeker, D.L. and Rothschild, M.F. 1987. Genetic control of immune responsiveness: a review of its use as a tool for selection for disease resistance. J. Anim. Sci. 64, 394-406.

Wassom, D.L. and Kelly, E.A.B. 1990. The role of the major histocompatibility complex in resistance to parasite infections. Crit. Rev. Immunol. 10, 31-52.

Wiegertjes, G.F., Daly, J.G. and van Muiswinkel, W.B. 1993a. Disease resistance of carp, *Cyprinus carpio* L.: identification of individual genetic differences by bath challenge with atypical *Aeromonas salmonicida*. J. Fish Dis. 16, 569-576.

Wiegertjes, G.F., Pilarczyk, A. and van Muiswinkel, W.B. 1995. Disease resistance and growth of two inbred carp (*Cyprinus carpio* L.) lines and their hybrid in pond culture. Aquacult. Research, in press.

Wiegertjes, G.F., Yano, T. and van Muiswinkel, W.B. 1993b. Estimation of the genetic variation in complement activity of common carp (*Cyprinus carpio* L.). Vet. Immunol. Immunopathol. 37, 309-319.

Withler, R.E. and Evelyn, T.P.T. 1990. Genetic variation in resistance to bacterial kidney disease within and between two strains of Coho salmon from British Columbia. Trans. Am. Fish. Soc. 119, 1003-1009.

Wolf, L.E. 1953. Development of disease resistant strains of fish. Trans. Am. Fish. Soc. 83, 342-349.

Xu, A., van Eijk, M.J.T., Park, C. and Lewin, H.A. 1993. Polymorphism in *BoLa-DRB3* exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. J. Immunol. 151, 6977-6985.

Zarnecki, A., Eide, D.M., Røed, K.H. and Rønningen, K. 1994. Immune traits as indicators for survival in Atlantic salmon (*Salmo salar*). In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph, Vol. XX, pp. 226-229.

Zijpp van der, A.J. and Nieuwland, M.G.B. 1986. Immunological characterization of lines selected for high and low antibody production. In: Proceedings of the 7th European Poultry Conference, Paris, pp. 211-215.

Chapter 2

Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis

Geert F. Wiegertjes, René J. M. Stet and Willem B. van Muiswinkel

Department of Experimental Animal Morphology and Cell Biology
Wageningen Agricultural University
P.O. Box 338, 6700 AH Wageningen, The Netherlands

Published in: **Animal Genetics 25, 251-257 (1994)**
Reproduced with permission of Blackwell Scientific Publications Ltd.

Summary

A base population (n=101) of carp, consisting of a single hybrid cross, was immunized with the hapten-carrier complex DNP-KLH, to perform a divergent selection for antibody response. Measurement of the DNP-specific antibody response at 12 and 21 days postimmunization, allowed the classification of a low number of individual carp as early/high (10%) or late/low (13%) responders. Three individuals defined as early/high and three defined as late/low responding, were gynogenetically reproduced to obtain corresponding homozygous progenies within one generation only. Upon immunization with DNP-KLH, the antibody response was found to be significantly higher in the early/high responder homozygous offspring. Although the homozygosity of the offspring apparently caused a (s)lower antibody response (compared with the base population), the differences between the high and low responder offspring do indicate a genetic influence on the antibody response. The realized heritability (h^2) for antibody production was estimated at 0.37±0.36. The present study provides the basis for a divergent selection of homozygous inbred carp lines with a genetically controlled difference in antibody response. These inbred lines will allow us to investigate relationship(s) between immune responsiveness and resistance to infectious diseases in fish.

Key words: *Cyprinus carpio* L., inbred lines, immune responsiveness, divergent selection

Introduction

Inbred laboratory fish lines, selected for high or low immune responsiveness, and in which these phenotypes have been clearly defined, can offer appropriate models for investigating the genetic factors of resistance to infections, similar to the role played by comparable mouse lines (Mouton *et al.*, 1988). The production of inbred fish lines, however, would take 15-20 generations, of 1.5-2 years each, if conventional sibmating is used (Falconer, 1989), or considerably more in those fish species, like common carp, that have been inferred to have a tetraploid status (Ohno *et al.*, 1967). Fortunately, the development of gynogenetic reproduction methods for poikilotherms, can accelerate the process of inbreeding in fish considerably (Streisinger *et al.*, 1981). These techniques have been developed for the common carp (Nagy *et al.*, 1978; Komen *et al.*, 1988; 1991), and application can result in inbred lines consisting of animals all genetically identical. They enable the production of an almost unlimited number of fish expressing the same unique genotype, within just two generations.

Such inbred lines can have a clear experimental value in unravelling environmental and genetic effects, and their stable genetic constitution can be invaluable for a thorough immunological analysis of disease resistance. They will allow comparison of data between subsequent generations or different laboratories. Our aim is to obtain inbred carp lines, generated from female individuals that have been divergently selected for immune responsiveness, to study the immunogenetic mechanisms behind disease resistance. To this end, similar to the work done

in mice (Biozzi *et al.*, 1979), antibody response was the immune parameter that served as the selection criterion.

We chose to study the primary antibody production to a hapten-carrier complex: dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH). The apparent genetic determination in mammals of the antibody response to simple hapten determinants, and correlation with immune response genes, mainly major histocompatibility complex (Mhc) class II (reviewed in Klein 1986), prompted the choice of a hapten-carrier complex as antigen. The antibody production was investigated in a base population of hybrid (female) carp. It was previously shown that progenies of the parental carp lines differed in resistance to the bacterium *Aeromonas salmonicida* (Houghton *et al.*, 1991), suggesting the existence of genetic differences in disease resistance within the base population. Three high-responding and three low-responding females were selected from the base population, and reproduced gynogenetically, obtaining homozygous progenies of which the immune responsiveness was verified.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were grown at 25±2°C or 23±2°C (isogenic carp only) in recirculation systems of filtered and UV-treated water. After initial feeding on *Artemia nauplii*, they were fed pelleted food (Trouvit, Trouw and Co., Putten, The Netherlands) at an age-dependent daily ration.

Isogenic hybrid carp, a cross between two homozygous parents (line E20 x E6; Komen *et al.*, 1991), were used to examine the effect of antigen leakage on the antibody response. At immunization, these fish were 17 months old with an average weight of 140 g.

The offspring of a hybrid cross (R3 x R8) between heterozygous parents, the female (No. 38) of Hungarian origin (R8 strain) and the male (No. 20) of Polish (R3 strain) origin, were used as the experimental base population to study the antibody response. The females (males were not used) were 13 months old and weighed 325 g on average at immunization.

Three high-responding and three low-responding individuals were chosen from the base population and gynogenetically reproduced by inhibiting the first mitotic division (Komen *et al.*, 1991), obtaining homozygous progenies. Fish of these progenies were immunized at the age of 6 months to verify their immune responsiveness. Their weights ranged from 41 to 90 g.

Carp of the base population were reared in the same tank starting after hatch. Their progenies were reared in the same recirculation system, and in the same tank one month prior to immunization onwards.

Immunization and detection of serum antibody

Fish were injected intramuscularly (i.m.) with 10 µg (except for the antigen-leakage experiment) $DNP_{494}KLH$ (Calbiochem, La Jolla, USA) in 50 µl phosphate-buffered saline (0.15M PBS, pH 7.2), mixed with 50 µl Freund's incomplete adjuvant (FIA; DIFCO, Detroit, USA). The antigen solution was injected in the lateral muscles on both sides of the animal, just under a dorsal scale to prevent outflow, using 0.5 ml 27-gauge tuberculin syringes (Sigma Chemical Company, St Louis, USA) for careful dosage application.

An enzyme-linked immunosorbent assay (ELISA) was used to detect DNP-specific serum antibodies. The assay and the buffer solutions were as described by Arkoosh and Kaattari (1990) with some modifications. In general, 96-well ELISA plates were washed twice for 10 s in tap water with 0.05% (v/v) Tween-20 (Merck, Schuchardt, Germany), in between all incubation steps. Solutions (100 µl) were incubated for 2 h at 37°C unless mentioned otherwise. Each plate contained a blank column (no serum) and serial dilutions of a standard immune serum sample to correct for plate differences. Plates were coated overnight at room temperature with 0.10 µg ml^{-1} $DNP_{44}BSA$ (dinitrophenyl-bovine serum albumin; Calbiochem, La Jolla, USA) in (bi)carbonate coating buffer (pH 9.6). Non-specific binding sites were blocked with 200 µl 3% (w/v) BSA (type IV, Boehringer, Mannheim, Germany) in tris-buffered saline (0.05M TBS, pH 8.0) containing 0.05% (v/v) Tween-20 detergent (T-TBS) for 45 min at 37°C. Diluted serum samples (1:100 in T-TBS) were added in triplicate. Next, a mouse monoclonal antibody (WCI12), specifically recognizing carp immunoglobulin (Secombes *et al.*, 1983; 1:500 in T-TBS) and subsequently goat anti-mouse horseradish peroxidase (GAM-HRP, BioRad, Richmond, USA; 1:2000 in T-TBS) was added. Orthophenylenediamine substrate solution (0.04% (w/v) OPD, 0.04% (w/v) hydrogen peroxide in 0.1M phosphate/0.2M citrate buffer) was added and the reaction stopped with 50 µl 2.5M sulfuric acid after 30 min incubation at room temperature. Optical density (OD) was determined at 492 nm (reference 690 nm) with a spectrophotometer (Anthos Reader 2001; Anthos Labtec Instruments, Salzburg, Austria).

Experimental design

First, nongenetic effects, such as the influence of antigen leakage after injection, on the magnitude and kinetics of the antibody response, and thus on a possible classification as high or low immune responsive, were investigated. Isogenic carp were injected with varying doses of antigen (5, 7.5 and 10 µg DNP-KLH/per animal), using the same volume (100 µl). Four fish per dose were bled weekly for a period of 6 weeks postimmunization, starting at 2 weeks.

For each selection step, all fish were of the same age, to exclude within-group differences due to age effects on the antibody response. Immunization procedures and treatments were kept the same for all animals. Variables that could not be controlled, such as weight and preimmune

antibody levels, were measured for each fish individually. A number of 101 female base population carp were marked by tattooing, and the antibody response, at 13 months, after immunization with 10 μg DNP-KLH was measured. High (n=3) and low (n=3) responding animals were set apart for gynogenetic reproduction. To verify their immune responsiveness, the resulting homozygous progenies were randomly sampled (n=9-23) and examined for the antibody response at 6 months of age, and not at 13 months, due to logistical constraints.

In general, fish were bled by caudal venipuncture after anaesthesia in 0.02% (‰) tricaïne methane sulphonate (TMS; Crescent Res. Chem., Phoenix, USA). Blood was allowed to clot at room temperature for 1 h to obtain serum which was stored at -80°C. Individual body weights were measured 4 weeks before (base population) or at immunization (homozygous progenies), to measure within-group weight differences.

The DNP-specific serum antibody levels were measured at 12, 21 or 28 (homozygous progenies only) days postimmunization. Preimmune DNP-reacting antibodies were measured 4 weeks before (base population) or at immunization (homozygous progenies).

Statistical significance ($P \leq 0.05$) of differences between progenies, in antibody titres (Student's t-test) or frequencies (χ^2 test) was tested using standard procedures (SAS Institute, 1990). Heritability was estimated as the ratio of selection response (R) and selection differential (S) (Falconer, 1989).

Results

Nongenetic effects

To study the effect of a loss of antigen at injection, on a putative classification as high or low immune responsive, isogenic carp were injected with different doses of DNP-KLH, mimicking 25-50% antigen loss. Intramuscular injection of 10 μg DNP-KLH, if mixed with FIA, resulted in an increase of the DNP-specific antibody response that could be measured by ELISA. No significant differences in either magnitude or kinetics of the antibody production, compared with an injection of 10 μg, were found injecting lower doses (data not shown). For further experiments, it was decided to inject 10 μg antigen, since the initial experiments demonstrated that even a considerable loss of antigen would not significantly influence the kinetics or height of the antibody response.

Antibody response base population

Measurement of the DNP-specific antibody response in the base population demonstrated significantly ($P \leq 0.0001$) increased optical density (OD) values at 12 and 21 days postimmunization (Table 1). The variation in antibody response was high, especially at 12 days (coefficient of variation (CV) 61%), as visualized by the frequency distributions (see Fig. 1a). Measurement of the antibody response at both 12 and 21 days allowed a classification not only based on the magnitude of antibody production, but also taking into account the kinetics of the response. Carp could be assigned to one of three groups, based on the individual DNP-specific antibody response (OD) at 12 and 21 days postimmunization, with arbitrary thresholds set at 1.0, 1.5 or 2.0 OD (Table 1). Some individuals (10%) could be grouped as early/high (12 days OD>1.5 and 21 days OD>2.0), others (13%) as late/low (12 days OD<1.0 and 21 days OD<1.5) responders. The OD values of the individuals belonging to these two defined groups are visualized in Fig. 1b. The remainder of the animals (77%) were considered to be medium responders.

Before immunization, medium to relatively high concentrations of serum antibodies reacting with the DNP determinant were observed in some animals (Fig. 1a). No significant correlation between preimmunization and postimmunization antibody levels was found however. A positive correlation (r=0.24) between body weight and antibody response was seen at 12 days, but not at 21 days, suggesting a slower but not a lower response in larger animals. Although no large effects of these variables on the antibody response were observed, only individuals with relatively low preimmune antibody levels and average body weights were chosen. Three representative early/high (Nos 17, 61, 69) and three late/low (Nos 51, 60, 85) responders from the base population were gynogenetically reproduced.

Table 1. Classification of early/high[1], late/low[2] and medium[3] antibody* responder carp from the base population

	Mean optical density (OD) ± SEM			
Group	Preimmune	12 days	21 days	*n*
All	0.35 ± 0.02	0.74 ± 0.04	2.03 ± 0.04	101
Early/high	0.38 ± 0.11	1.67 ± 0.03	2.28 ± 0.04	10
Medium	0.33 ± 0.02	0.68 ± 0.04	2.14 ± 0.03	78
Late/low	0.43 ± 0.09	0.38 ± 0.06	1.13 ± 0.11	13

[1] Early/high: 12 days OD>1.5 and 21 days OD>2.0; [2] Late/low: 12 days OD<1.0 and 21 days OD<1.5; [3] Medium: remainder; * Immunizations were with DNP-KLH, serum antibodies (1:100) were measured by ELISA, before immunization, and 12 and 21 days after immunization.

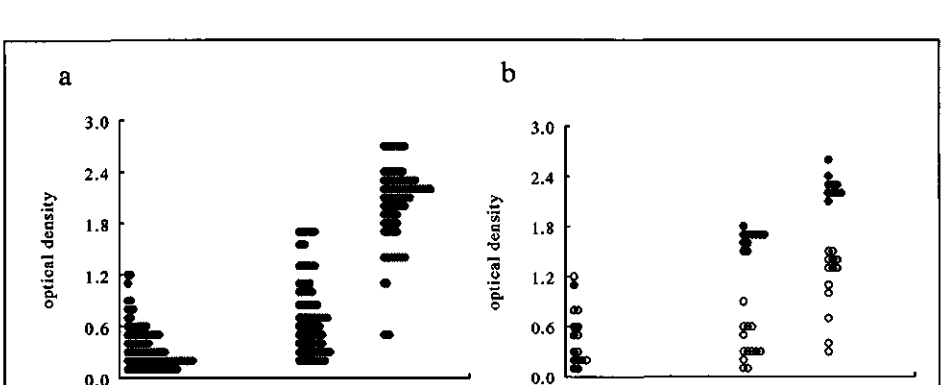


Figure 1. Antihapten antibody response (optical density OD), before immunization, and 12 and 21 days after immunization of carp with DNP-KLH. **a.** Frequency distributions of the antibody response in the hybrid base population. The length of each line is proportional to the percentage of animals with the same optical density. **b.** Antibody responses of individuals from the base population classified as early/high (12 days OD>1.5 and 21 days OD>2.0) or late/low (12 days OD<1.0 and 21 days OD<1.5) responder.

Table 2. Antibody response* of the homozygous progenies, gynogenetically produced from early/high and late/low responder carp selected from the base population

Group	Mean optical density (OD) ± SEM				
No. parent	Preimmune	12 days	21 days	28 days	*n*
Early/high responder progenies					
17	0.39 ± 0.07	0.30 ± 0.05	1.04 ± 0.13	1.56 ± 0.16	10
61	0.41 ± 0.04	0.36 ± 0.04	0.86 ± 0.11	1.33 ± 0.12	23
69	0.69 ± 0.09	0.37 ± 0.06	0.91 ± 0.21	1.95 ± 0.16	9
Late/low responder progenies					
51	0.44 ± 0.12	0.58 ± 0.13	0.81 ± 0.17	1.15 ± 0.19	13
60	0.48 ± 0.07	0.45 ± 0.04	1.00 ± 0.09	1.27 ± 0.10	14
85	0.62 ± 0.13	0.21 ± 0.03	0.74 ± 0.12	1.25 ± 0.13	14

* Immunizations were with DNP-KLH, serum antibodies (1:100) were measured by ELISA, before, and 12, 21 and 28 days after immunization.

Antibody response homozygous progenies

Random samples of 9-23 fish, were taken from the early/high and late/low responder homozygous progenies, and immunized with DNP-KLH. In contrast to the results of the base population, the antibody response (OD) of the homozygous progenies at 12 days postimmunization, showed no significant increase compared with preimmunization levels (Table 2). At 21 days, however, antibody levels were significantly higher in all progenies but those of fish No. 51. Owing to the apparent slower (or lower) response of the homozygous fish, the titres at 28 days were also included in the analysis. A further increase in antibody levels was observed, except for the progeny of fish No. 51, which showed a gradual increase. The variability of the antibody response within each homozygous progeny was very high, as shown by CVs of up to 75% (progeny No. 51, 21 days).

Individuals were assigned to one of three groups, based on their DNP-specific antibody response (OD) at both 21 and 28 days postimmunization, with arbitrary thresholds set at 0.5, 0.75, 1.0 or 1.5 OD. Similar to the base population, within each progeny, certain individuals could be grouped as high (21 days OD>0.75 and 28 days OD>1.5), and others as low (21 days OD<0.5 and 28 days OD<1.0) responding. The remainder were considered medium responders. The frequencies of high, low and medium responders within the progenies are shown in Figure 2. The homozygous progenies resulting from (base population) carp previously characterized as early/high responder (Nos 17, 61, 69) contained significantly ($P \leq 0.001$) higher percentages of high responding, and lower percentages of medium ($P \leq 0.004$) responding offspring (thresholds as defined above), than the late/low responder (Nos 51, 60, 85) homozygous progenies. The frequencies of low-responding offspring were not significantly different between the two types of progenies. No significant differences in weight or preimmune antibody concentrations were observed between the high- and low-responder progenies.

Genetic effects

The difference in weighted OD values of the homozygous progenies, at 28 days, indicated a significantly ($P \leq 0.05$) higher response in the homozygous high responder offspring compared with the low responder offspring (Table 2). This resulted in a selection response (R) of 0.29±0.25 OD, based on the weighted means of the parents and offspring. Dividing the selection response by the selection differential (S) of 0.79±0.08 OD (see Table 1), estimates a realized heritability (h^2) of 0.37±0.36.

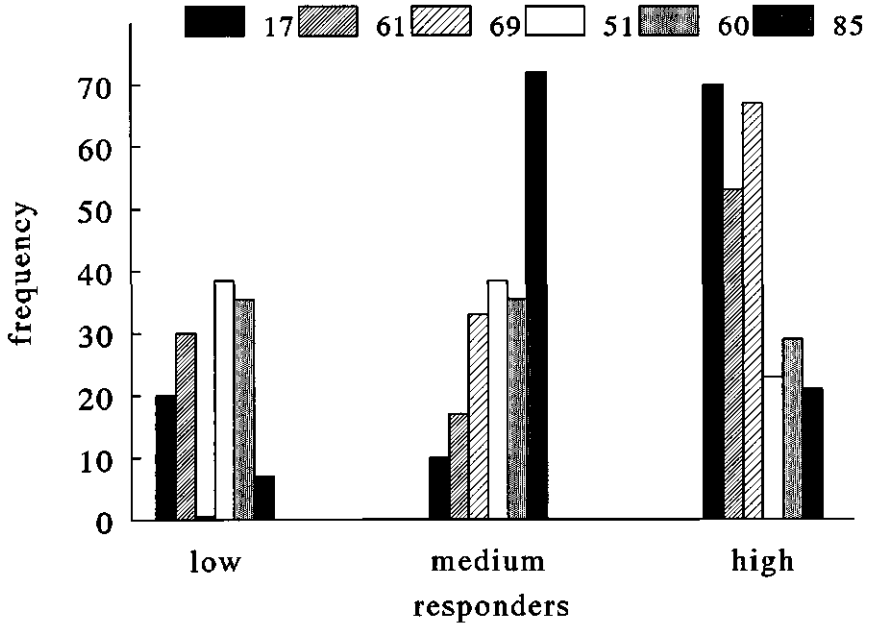


Figure 2. Frequencies of low (21 days OD<0.5 and 28 days OD<1.0), high (21 days OD>0.75 and 28 days OD>1.5), and medium (remainder) responding animals, after DNP-KLH immunization. Carp were the homozygous offspring of six individuals selected from the base population as either early/high (Nos 17, 61, 69) or late/low (Nos 51, 60, 85) responder.

Discussion

To minimize differences in environmental effects on the antibody response, carp were of the same age, and were reared and treated comparably. It was shown that, with a dose of 10 μg antigen, no significant differences in the kinetics or magnitude of the antibody response can be expected after an unnoticed loss of antigen at the injection site(s). Although no large effects of weight or preimmune titres on the antibody response were observed, only individuals with relatively low preimmune antibody levels and average body weights were selected. The observed medium to high levels of spontaneous nitrophenyl-reacting antibodies, were previously noticed by Vilain *et al.* (1984) to be a common phenomenon for several cyprinid fish species. Recent measurements in a particular homozygous inbred line, demonstrated all carp tested for anti-DNP response (n=27), to have high (1.0 OD) preimmune antibody levels (unpublished data), suggesting a genetic determination of preimmune DNP-titres. This would allow for a selection of homozygous inbred lines with, genetically determined, low preimmune titres.

Differences in kinetics and magnitude of the antibody response, resulted in the classification of a limited number of carp as early/high (10%) or late/low (13%) responders. Three early/high and three late/low responders were gynogenetically reproduced through inhibition of the first mitosis, and homozygous progenies were obtained. At this step in the development of inbred lines, fish are homozygous, but owing to a segregation of genes, all are expected to be different. It is important to realize that the low hatching percentages (5-10%) that are considered normal for this gynogenetic technique (Komen *et al.*, 1991), and are probably caused by the additive effect of egg-handling and lethal combinations of genes, impose a strong selection pressure to act on such homozygous progenies.

The antibody levels of the homozygous progenies were highly variable, probably due to the expected segregation of homozygous (combinations of) alleles influencing the antibody response. Furthermore, in general, the homozygous fish showed a (s)lower response than the hybrid carp from the base population, which might be due to inbreeding depression caused by homozygous genotypes negatively influencing the antibody response. In addition, the absence of heterozygous advantage on the antibody response might be such that lowered fitness in the homozygous fish will result in a different antibody response, possibly putting natural selection in opposition to the artificial selection (Lerner, 1954). Further results on the selection may give more information about this phenomenon in fish.

The verification of responsiveness was done in small 6-month-old fish and not, similar to the base population, in 13-month-old fish, due to logistical constraints. It does not seem likely that the age difference at immunization between the fish of the base population and their progenies, caused the (s)lower antibody response. Recent observations on the antibody response of one high responder (No. 69) and one low responder (No. 85) progeny, suggest a (s)low antibody response at 12 months also, with a similar difference in response between the two groups. Also, Koumans-van Diepen *et al.* (1994) observed that the development of plasma cells in carp can reach a plateau at 3 months of age.

Despite the apparent (s)lower response, the results again allowed a classification of high- and low-responding individuals, within the homozygous progenies. The early/high-responder progenies indeed contained more animals with a high response, compared with the late/low-responder progenies. The latter progenies had significantly more medium-responding animals. The realized heritability (h^2) of 0.37±0.36, suggests an inheritance of the magnitude of the antibody response. Also, previous observations in rainbow trout (*Oncorhynchus mykiss*), on the primary antibody response to DNP-KLH, showed less variable antibody titres in fish obtained by self-fertilization (Cossarini-Dunier *et al.*, 1986). These results indicate a genetic determination of the primary antibody response in fish, allowing for a successful selection procedure. The next step for obtaining high and low antibody responder inbred lines, will be the gynogenetic

reproduction of such individuals chosen from the high- and low-responder homozygous progenies.

The successful selection in mice (Biozzi *et al.*, 1979) of high and low antibody responder lines was explained by several genetic differences between the two lines. They estimated that a number of 10 independent loci regulated the antibody response, one of which was *H-2* (probably Mhc class II)-linked and contributed 10-18% of the total difference between the lines. The complexity of the genetic regulation, however, decreased with the dose of antigen administered, the antibody response then being directed towards the most immunogenic determinant. Injection of threshold instead of optimal doses of erythrocytes suggested 61% of the interline difference to be *H-2*-linked. Results obtained by Freed *et al.* (1976), who used a classical hapten-carrier system (DNP-conjugates of ovomucoid or bovine γ-globulin) to study the antibody response in a number of inbred mouse strains, suggested a primary control of the hapten-specific antibody response by immune response genes.

The recent discovery of a putative Mhc in carp (Hashimoto *et al.*, 1990), allowed the description of restriction fragment length polymorphism with class II-specific probes (Stet *et al.*, 1993), and sequence polymorphism in the peptide-binding region (PBR) of class II molecules (Ono *et al.*, 1993). The class II PBR polymorphism, together with the divergent selection for antibody response, might enable us to establish a possible relationship between immune responsiveness and functional Mhc class II alleles in the base population.

Acknowledgements

The authors would like to thank A. Hutten of the central fish facilities complex "De Haar Vissen" for taking care of the carp. The technical assistance of C. Berkhout and A. Groeneveld is highly appreciated. Furthermore, we wish to thank dr. E. Egberts for critically reading the manuscript, and the people from the Department of Animal Breeding for helpful discussions on the genetic effects.

References

Arkoosh, M.R. and Kaattari, S.L. 1990. Quantitation of fish antibody to a specific antigen by an enzyme-linked immunosorbent assay. In: Techniques in fish immunology (eds: Stolen, J.S., Fletcher,T.C., Anderson, D.P., Roberson, B.S. and van Muiswinkel, W.B.). SOS Publ., Fair Haven, NJ, USA, pp. 15-23.

Biozzi, G., Mouton, D., Sant'Anna, O.A., Passos, H.C., Gennari, M., Reis, M.H., Ferreira, V.C.A., Heumann, A.M., Bouthillier, Y., Ibanez, O.M., Stiffel, C. and Siquera, M. 1979. Genetics of immunoresponsiveness to natural antigens in the mouse. Curr. Top. Microbiol. Immunol. 85, 31-98.

Cossarini-Dunier, M., Desvaux, F-X. and Dorson, M. 1986. Variability in humoral responses to DNP-KLH of rainbow trout (*Salmo gairdneri*). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis and self-fertilization. Dev. Comp. Immunol. 10, 207-217.

Falconer, D.S. 1989. Introduction to quantitative genetics. John Wiley & Sons Inc., New York.

Freed, J.H., Deak, B.D. and McDevitt, H.O. 1976. Mapping of the genetic control of murine response to low doses of the dinitrophenyl conjugates of ovomucoid and bovine γ-globulin. J. Immunol. 117, 1514-1518.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. 1990. Isolation of carp genes encoding major histocompatibility complex genes. Proc. Nat. Ac. Sci. USA 87, 6863-6867.

Houghton, G., Wiegertjes, G.F., Groeneveld, A. and van Muiswinkel, W.B. 1991. Differences in resistance of carp, *Cyprinus carpio* L., to atypical *Aeromonas salmonicida*. J. Fish Dis. 14, 333-341.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley & Sons, New York.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman, E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.) II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

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.

Koumans-van Diepen, J.C.E., Taverne-Thiele, J.J., van Rens, B.T.T.M. and Rombout, J.H.W.M. 1994. Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. Fish Shellf. Immunol. 4, 19-28.

Lerner, I.M. 1954. Genetic homeostasis. Oliver & Boyd, London.

Mouton, D., Sant'Anna, O.A. and Biozzi, G. 1988. Multigenic control of specific and non-specific immunity in mice. A review. Livest. Prod. Sci. 20, 277-286.

Nagy, A., Rajki, K., Horváth, L. and Csányi, V. 1978. Investigation on carp, *Cyprinus carpio* L. gynogenesis. J. Fish Biol. 13, 215-224.

Ohno, S., Muramoto, J., Christian, L. and Atkin, N.B. 1967. Diploid-tetraploid relationship among old-world members of the fish family *Cyprinidae*. Chromosoma 23, 1-9.

Ono, H., O'hUigin, C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. 1993. New β chain-encoding Mhc class II genes in carp. Immunogenetics 38, 146-149.

SAS Institute Inc. 1990. SAS User's Guide: Statistics. Version 6, 4th edn, Vol 2. SAS Institute Inc., Cary, NC.

Secombes, C.J., van Groningen, J.J.M. and Egberts, E. 1983. Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immunohistochemical studies. Immunology 48, 165-175.

Stet, R.J.M., van Erp, S.H.M., Hermsen, T., Sültmann, H.A. and Egberts, E. 1993. Polymorphism and estimation of the number of *MhcCyca* class I and class II genes in laboratory strains of the common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 17, 141-156.

Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291, 293-296.

Vilain, C., Wetzel, M-C., Du Pasquier, L. and Charlemagne, J. 1984. Structural and functional analysis of spontaneous anti-nitrophenyl antibodies in three cyprinid fish species: carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tench (*Tinca tinca*). Dev. Comp. Immunol. 8, 611-622.

Chapter 3

Divergent selection for antibody production to produce standard carp (*Cyprinus carpio* L.) lines for the study of disease resistance in fish

Geert F. Wiegertjes, René J. M. Stet and Willem B. van Muiswinkel

Department of Experimental Animal Morphology and Cell Biology
Wageningen Agricultural University
P.O. Box 338, 6700 AH Wageningen, The Netherlands

Aquaculture (1995): in press

Summary

Carp with an early/high or a late/low antibody response were reproduced gynogenetically, resulting in homozygous progenies. After an initial test of their immune responsiveness at 6 months of age, we chose to continue the selection procedure with high responder progeny No. 69 and low responder progeny No. 85. The antibody response of these groups, at 12 months of age, was shown to be consistent with the parental responder type. The realized heritability (h^2) was estimated at 0.29. Progeny No. 69 showed a higher antibody response than progeny No. 85, both at 21 and at 28 days after immunization with dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH). The antibody response in progeny No. 85 did not significantly increase after 21 days, in accordance with this group's classification as late/low responders. Furthermore, a significantly lower percentage (11%) of the animals of this group were early/high responders, compared with progeny No. 69 (45%). Late/low responders, however, were found in almost equal numbers in both progenies. The possibility to reproduce both females and functional males with a high or a low antibody response, will result in standardized fish lines that might be important for experimental studies on the immunogenetics of disease resistance.

Key words: *Cyprinus carpio* L., immune responsiveness, inbred lines, disease resistance

Introduction

Indirect selection criteria for disease resistance, such as immune response parameters, can lead to individual identification of potentially healthy fish, and their advantage is that difficult experimental infection models, necessary for direct selection schemes, are not needed (Chevassus and Dorson, 1990). However, although significant genetic variation has been found in immune responses, more knowledge is needed on the genetic correlations of immune parameters with disease survival, before these responses can be used for indirect selection (Fjalestad *et al.*, 1993). Previously, inbred mouse lines divergently selected for immune responsiveness, have offered appropriate models for investigating the genetic factors of resistance to infection (Mouton *et al.*, 1988). Therefore, inbred fish lines, resulting from a bidirectional selection for antibody response, could have a clear experimental value in unravelling environmental and genetic effects on disease resistance in fish.

Our aim is to apply gynogenetic techniques to generate inbred carp lines from females that have been selected for high or low antibody responsiveness. For this purpose, the following selection strategy has been adopted. From the individuals of a hybrid base population that were characterized as either early/high or late/low antibody responders to DNP-KLH (approximately 10% of the base population, each), three females from each group were reproduced gynogenetically. The immune responsiveness of these homozygous progenies was measured at 6 months of age (Wiegertjes *et al.*, 1994), and based on these results, the selection procedure was continued with one early/high responder progeny (No. 69), and one late/low responder progeny (No. 85).

These fish were immunized with DNP-KLH at a similar age as the base population (12 months), in order to use the same selection criterium. The results from this immunization are described, and the possibilities to produce standardized carp lines are discussed.

Materials and methods

Animals and selection scheme

Common carp (*Cyprinus carpio* L.) were grown at 25±2°C in recirculated, filtered and UV-treated water. After initial feeding on *Artemia nauplii* for a period of 3 weeks following yolk-sac absorption, the feeding regime was switched to a daily ration of 2% body weight (Trouvit, Trouw and Co., Putten, The Netherlands). The homozygous carp were reared in the same system starting post-hatch, and held in the same tank from one month before immunization.

The experimental base population consisted of the female offspring of a hybrid cross (R3 x R8, n=101) between a female of Hungarian origin (R8 strain) and a male of Polish (R3 strain) origin. From this base population, six females, chosen for their high or low immune responsiveness, were gynogenetically reproduced by inhibiting the first mitotic division, such as described by Komen *et al.* (1991). Fish taken at random from the resulting homozygous progenies, were immunized at the age of 6 months, to measure their immune responsiveness (Wiegertjes *et al.*, 1994).

Subsequently, two of these homozygous progenies, one with a high antibody response (No. 69, n=83) and one with a low antibody response (No. 85, n=95), were chosen for further characterization. The average weights at immunization (12 months) of these groups were 178±69 g (No. 69) and 166±36 g (No. 85), respectively.

Immunization and detection of serum antibody

Fish were injected intramuscularly with 10 μg dinitrophenyl-keyhole limpet haemocyanin ($DNP_{494}KLH$; Calbiochem, La Jolla, USA) in Freund's incomplete adjuvant. A previously described, and only slightly adjusted, enzyme-linked immunosorbent assay (ELISA) was used to detect DNP-specific serum antibodies (Wiegertjes *et al.*, 1994). Briefly, 96-well ELISA plates were coated overnight with 0.10 μg ml^{-1} dinitrophenyl-bovine serum albumin ($DNP_{44}BSA$; Calbiochem, La Jolla, USA) at 37 °C, and incubated with BSA to block non-specific binding sites. Diluted serum samples (1:100) were added in triplicate. Then, plates were incubated with WCI-12, a mouse monoclonal antibody against carp immunoglobulin (Secombes *et al.*, 1983), and subsequently with goat-anti-mouse-Ig-horseradish peroxidase (BioRad, Richmond, USA). Orthophenylenediamine substrate incubation was stopped with sulphuric acid, and the optical density (OD) determined with a spectrophotometer (492 nm).

Fish were bled by caudal venepuncture after anaesthesia in 0.02% (w/v) Tricaine Methane Sulphonate (Crescent Research Chemicals, Phoenix, AZ). Blood was allowed to clot at room temperature for 1 h to obtain serum which was stored at -20°C. Pre-immune DNP-specific serum antibody levels, and levels 12, 21 and 28 days after immunization were determined. Individual weights were measured at immunization.

Statistical analysis

Statistical comparison ($P \leq 0.05$) of differences in antibody titres (Student's t test) and frequencies (χ^2 test), between progenies, was done using standard procedures (Statistical Analysis Systems Institute Inc., 1990).

Results and discussion

The antibody response of the two selected homozygous progenies (No. 69 and No. 85), at 12 months of age, is shown in Table 1. For comparison, the antibody responses of these progenies at 6 months immunization, and the response of the individual parents, are also included in this table. Although the fish of both progenies were immunized at 12 months, comparable to the age of the base population at immunization (13 months), the onset of the antibody response in the homozygous animals was somewhat delayed (Table 1). This phenomenon however, was less obvious than at the measurements at 6 months of age. Apparently, between 6 and 12 months, the immune system of these carp was still developing. Indeed, Koumans-van Diepen *et al.* (1994), observed changes in the B cell compartments with age, and suggested that the humoral immune system of carp raised at 21 °C, reaches a plateau in development at 8 months.

In addition, the height of the antibody response of the homozygous progenies, at 12 months, seemed somewhat reduced compared with the response of the base population carp (Table 1). It should be realized, however, that the ELISA measurements were corrected within, but not between generations, which renders a comparison of these results difficult. The inbred nature of the progenies may have influenced the response. However, although inbreeding depression often results in lowered fitness, the exact nature of the effects of inbreeding on the immune response is still unknown.

The above mentioned suggestion of a (s)lower antibody response, however, did not influence the characterization of the respective homozygous progenies as high or low responsive. The early/high responder offspring (No. 69) had higher antibody values, both at 21 ($P \leq 0.001$) and at 28 days ($P \leq 0.001$), than the late/low responders (No. 85). This confirmed the earlier observations at 6 months of age (Wiegertjes *et al.*, 1994), and again demonstrated the antibody response of the homozygous progenies to be consistent with the parental responder type.

In both progenies, antibody levels significantly ($P \leq 0.01$) increased at 21 days (compared with pre-immune levels), and in the early/high responder offspring (No. 69), antibody levels increased further at 28 days ($P \leq 0.01$). Antibodies in progeny No. 85 however, showed no significant increase at 28 days, which is in accordance with the definition of progeny No. 85 as late/low responders. A small, but significant ($P=0.04$), difference in pre-immune values was observed between both groups, but on average, neither group showed high pre-immune titres (Table 1). Also, differences in body weight could not explain the differences in antibody response between the progenies.

Applying the same optical density (OD) thresholds used for the base population, again early/high (21 days OD>1.5 and 28 days OD>2.0) and late/low (21 days OD<1.0 and 28 days OD<1.5) responding individuals could be defined. Within progeny No. 69, a significantly ($P \leq 0.0001$) higher percentage (45%) of the animals was defined as early/high responding, compared with progeny No. 85 (11%). The difference in frequency distribution (Fig. 1), skewed the antibody response of progeny No. 69 in the direction of higher responsiveness, especially at 28 days. Although the frequency of late/low responders differed significantly ($P \leq 0.05$) between both progenies (progeny No. 69; 12% versus progeny No. 85; 17%), the difference was small. Indeed, with divergent selection procedures, the magnitude of genetic change is often not equal for selection in upward and downward direction. This may be a matter of scaling, especially when the plateau is reached first in downward direction, or it may be related to the confounding of selection and inbreeding effects (Lerner, 1954).

The present data lead to a realized heritability (h^2) of 0.29, calculated as the ratio of the weighted difference in selection (=antibody) response of the homozygous progenies (R=0.25), and the selection differential of the parents (S=0.86) (Falconer, 1989). Although this figure is slightly lower than the $h^2=0.37$ estimated on the basis of the 6 months data (Wiegertjes *et al.*, 1994), it again suggests a clear genetic influence on the antibody response.

The complete absence of heterozygosity in the gynogenetic progenies can be expected to have a negative effect on fitness, and indeed, Komen *et al.* (1992b) observed considerable inbreeding depression in the gonad development of homozygous gynogenetic carp, resulting in among others a retarded vitellogenesis and a reduced ovulation response. Occasionally however, individual carp with genotypes less negatively influenced by homozygosity were found (Komen *et al.*, 1992b). Therefore, the gonad development of each homozygous individual, at 16 months of age, was examined by testing the reproduction capacity after injections with pituitary suspension. It was shown that the gynogenetic reproduction of animals with an early/high, but also of animals with a late/low antibody response might be possible (A.B.J. Bongers, unpublished observations, 1994).

Table 1. Antibody response (optical density) after immunization of carp with DNP-KLH. The response was measured by ELISA, before, and 12, 21 and 28 days after immunization.

No.	Group	Age (months)	mean optical density ± SE				*n*
			pre-immune	12 days	21 days	28 days	
69	parent (base population)	13	0.11	1.73	2.23	nd	1
	homozygous progeny	6	0.69±0.09	0.37±0.06	0.91±0.21	1.95±0.16	9
	homozygous progeny	12	0.32±0.03	0.53±0.03	1.65±0.06	1.81±0.05	83
85	parent (base population)	13	0.04	0.86	1.37	nd	1
	homozygous progeny	6	0.62±0.13	0.21±0.03	0.74±0.12	1.25±0.13	14
	homozygous progeny	12	0.25±0.02	0.37±0.02	1.39±0.04	1.56±0.04	95

nd = not done

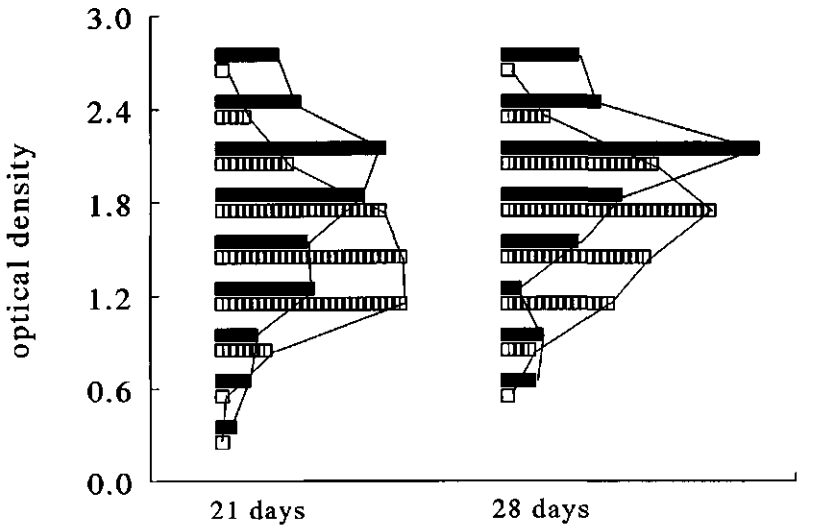


Figure 1. Frequency distributions of the antibody response (optical density) at 12 months of age, of early/high responder progeny No. 69 (■) and late/low responder progeny No. 85 (□). The response visualized was measured at 21 and 28 days after immunization with DNP-KLH. The height of each column is proportional to the percentage of animals with the same optical density.

Since the individuals are homozygous, such inbred lines are expected to show an immune responsiveness similar to their parent. In response to the injections with pituitary suspension, within both progenies, approximately 10% of the animals, including both some high and low responders, were seen to produce milt. Previously, homozygous progenies of carp, produced by the same methodology, have been shown to contain functional males with the XX genotype (Komen *et al.*, 1992a). Such phenotypic males might be used to produce stable high or low responding hybrid lines, exploiting hybrid vigour. The high or low responder male phenotype can be reproduced by the use of androgenetic techniques recently developed for carp (Bongers *et al.*, 1994).

One of the genes or gene-complexes which may play a major role in determining the antibody response to DNP-KLH in our carp lines, could be the Major Histocompatibility Complex (Mhc). The possibility to analyze sequence polymorphism in the peptide binding region of *MhcCyca-DAB* class II molecules (Ono *et al.*, 1993), and the availability of frozen DNA samples from all individuals from the base population, might allow the study of a correlation between the presence of Mhc class II immune response genes, and antibody response capacity. Moreover, the association of Mhc polymorphism and disease resistance, which has received much attention in other livestock species (Andersson, 1994), but little or none in fish, may then be feasible.

In conclusion, we expect that the standardized carp lines that will result from the above described selection scheme, allow an investigation of the role of the humoral response in the defence against various pathogens, which may reveal the level of antibody production to be an indirect marker for disease resistance in fish.

Acknowledgements

This research was financially supported by the Netherlands Technology Foundation (STW), and was coordinated by the Life Sciences Foundation (SLW). The authors would like to thank A. Hutten of "De Haar Vissen" for taking care of the carp, and A. Groeneveld for technical assistance. We wish to thank dr. J.A.M. van Arendonk from the Department of Animal Breeding for helpful discussions on the genetic effects, and dr. C.J.J. Richter from the Department of Fish Culture and Fisheries for critically reading the manuscript.

References

Andersson, L. 1994. The role of Mhc polymorphism in disease/parasite resistance. In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph, Vol. XXI, pp 177-182.

Bongers, A.B.J., in 't Veld, E.P.C., Abo-Hashema, K., Bremmer, I.M., Eding, E.H. and Richter, C.J.J. 1994. Androgenesis in common carp (*Cyprinus carpio* L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture 122, 119-132.

Chevassus, B. and Dorson, M. 1990. Genetics of resistance to disease in fishes. Aquaculture 85, 83-107.

Falconer, D.S. 1989. Introduction to quantitative genetics. Jogn Wiley & Sons Inc., New York.

Fjalestad, K.T., Gjedrem, T. and Gjerde, B. 1993. Genetic improvement of disease resistance in fish: an overview. Aquaculture 111, 65-74.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman, E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.) II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

Komen, J., de Boer, P. and Richter, C.J.J. 1992a. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio* L.) by a recessive mutation in a sex-determining gene. J. Hered. 83, 431-434.

Komen, J., Wiegertjes, G.F., van Ginneken, V.J.T., Eding, E.H. and Richter, C.J.J. 1992b. Gynogenesis in common carp (*Cyprinus carpio* L.). III. The effects of inbreeding on gonadal development of heterozygous and homozygous gynogenetic offspring. Aquaculture 104, 51-66.

Koumans-van Diepen, J.C.E., Taverne-Thiele, J.J., van Rens, B.T.T.M. and Rombout, J.H.W.M. 1994. Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. Fish Shellf. Immunol. 4, 19-28.

Lerner, I.M. 1954. Genetic homeostasis. Oliver & Boyd, London.

Mouton, D., Sant'Anna, O.A. and Biozzi, G. 1988. Multigenic control of specific and non-specific immunity in mice. A review. Livest. Prod. Sci. 20, 277-286.

Ono, H., O'hUigin, C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. 1993. New β chain-encoding *Mhc* class II genes in carp. Immunogenetics 38, 146-149.

SAS Institute Inc. 1990. SAS User's Guide: Statistics. Version 6, 4th edn, Vol 2. SAS Institute Inc., Cary, NC.

Secombes, C.J., van Groningen, J.J.M. and Egberts, E. 1983. Separation of lymphocyte subpopulations in carp (*Cyprinus carpio* L.) by monoclonal antibodies: immunohistochemical studies. Immunology 48, 165-175.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1994. Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. Anim. Genet. 25, 251-257.

Chapter 4

Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.)

Geert F. Wiegertjes, A. Groeneveld and Willem B. van Muiswinkel

Department of Experimental Animal Morphology and Cell Biology
Wageningen Agricultural University
P.O. Box 338, 6700 AH Wageningen, The Netherlands

Published in: **Veterinary Immunology and Immunopathology 47, 153-161 (1995)**

Summary

Gynogenetic reproduction of homozygous females, or crossbreeding two homozygous animals, results in fish lines without genetic variation. Hybrid crosses are expected to express a more stable development than homozygous lines, the latter may have an important value for gaining insight into genetic components of host resistance to parasite infection. We examined the antibody response of carp (*Cyprinus carpio* L.) to infection with *Trypanoplasma borreli*. Outbred carp responded with a production of specific antibodies, but highly susceptible isogenic hybrid carp did not. This suggests an apparent relationship between susceptibility and the lack of specific antibody production. This relation was partially confirmed by the passive transfer of immunity with immune plasma. In addition, two isogenic homozygous carp lines were highly susceptible to the trypanoplasm (100% mortality), in contrast with outbred carp, the majority of which survived infection. None of the carp in either homozygous carp line produced an antibody response to parasite-unrelated antigen (DNP-KLH). This suggests that the low antibody response was not entirely due to a poor state of health, but that these carp have a genetically predetermined low antibody response.

Key words: *Cyprinus carpio* L., isogenic lines, disease model, genetic variation

Introduction

Trypanoplasma borreli (Kinetoplastida: Cryptobiidae) is a parasite of European cyprinids (Lom, 1979), of which some strains cause mortality in common carp, *Cyprinus carpio* L. Jones *et al.* (1993) developed a laboratory model to monitor antibody levels in outbred carp, following infections with *T. borreli*, and found the significance of differences between various infective doses to be low because of the high variability in parasitaemia. The peak antibody response in these carp coincided with a decline and eventual absence of parasitaemia, resulting in the survival of the majority of fish. However, the exact nature of the immune mechanism responsible for eliminating the parasite remains unclear.

The use of fish as research animals is limited by the extreme variation commonly encountered. Isogenic lines, either homozygous, or hybrid F_1s between two homozygous individuals, can reduce this variation. Murine inbred lines, and possibly homozygous fish lines, are essential tools for the analysis of inheritance of susceptibility to parasite infection, and can facilitate detection of characters under single gene control (Festing and Blackwell, 1988). Hybrid F_1s between homozygous fish are free of recessive deleterious genes and show hybrid vigour with a reduction in variation (Streisinger *et al.*, 1981). Hybridization may result in fish lines highly suitable for biological assays.

The application of artificial gynogenesis results in the rapid production of isogenic fish lines. In this technique, the elimination of the genetic contribution of the sperm, and subsequent diploidisation of the 'fertilized' egg at the moment of first mitotic division, results

in homozygous animals in only one generation. Subsequently, isogenic lines can be obtained by gynogenetic reproduction of homozygous females, or by crossbreeding two homozygous animals. Homozygous individuals have been produced by gynogenesis in a number of fish species, but established homozygous lines have only been reported for zebra fish (*Brachydanio rerio*: Streisinger *et al.*, 1981; Hörstgen-Schwark, 1993), medaka (*Oryzias latipes*: Naruse *et al.*, 1985), ayu (*Plecoglossus altivelis*: Han *et al.*, 1991), and carp (Komen *et al.*, 1991).

In the present study we used isogenic hybrid F_1 carp to investigate the role of the antibody response in resistance to *T. borreli*. Furthermore, we examined the susceptibility of two homozygous carp lines to infection with *T. borreli*, and their ability to mount an antibody response to *T. borreli* or to a hapten-carrier complex.

Materials and Methods

Animals

A single, homozygous, offspring was obtained by gynogenetic reproduction (inhibition of the first mitotic division) of an outbred female carp (*Cyprinus carpio* L.). Two females from this offspring (Nos 4 and 20), were reproduced gynogenetically (by retention of the second polar body) to produce two homozygous lines (E4 and E20, respectively). A homozygous phenotypical male (E6) from the same offspring, was crossed with the homozygous female No. 20 to produce a hybrid F_1 (E20 x E6), as described by Komen *et al.* (1991). Outbred carp were obtained by either mixing sperm from different males to fertilize a mixture of eggs, or by mixing carp from different strains.

Carp, 1-2 years old and weighing 100-300 g, were reared at 23±2°C in recirculated, filtered and UV-treated tap water. After initial feeding on *Artemia nauplii* for 3 weeks following yolk-sac absorption, the feeding regime was switched to a daily ration of pelleted trout food (Trouvit: Trouw and Co., Putten, The Netherlands) of 25 g kg^{-1} metabolic weight. Before infection with *T. borreli*, carp were acclimated to a flow-through system of separate 120 l aquaria at 20±2°C, for at least 2 weeks. Fish were anaesthetized in 0.02% (w/v) Tricaïne Methane Sulphonate (Crescent Research Chemicals, Phoenix, AZ) solution, before blood samples were taken by caudal venepuncture.

Parasite infection

Trypanoplasma borreli was cloned and characterized by Steinhagen *et al.* (1989), and maintained by syringe passage through highly susceptible carp, as described by Jones *et al.* (1993). Hybrid F_1 (E20 x E6) as well as outbred carp (n=24 per group), were infected i.m. with 5 x 10^4 *T. borreli* (Jones *et al.*, 1993). Parasitaemia and antibody production were

measured in blood samples taken weekly from two randomly assigned fish of each group, which were subsequently returned to the system. All fish were also bled before infection. Parasitaemia was monitored using a Bürker counting chamber. Serum samples for antibody measurements were obtained from fresh blood samples, after clotting at room temperature for 1 hour, and stored at -20 °C.

Two homozygous carp lines (E4, E20), and outbred carp (n=10 per group; in duplicate), were infected i.m. with 100 *T. borreli*. The low infection dose was used to lengthen the prepatent period (Jones *et al.*, 1993), because of the high sensitivity of siblings of the parental carp (S.R.M. Jones, unpublished observations, 1991). Dead fish were removed daily for 5 weeks post-infection (w.p.i.).

Passive immunization

Susceptible hybrid carp (E20 x E6, n=4 per group) were infected i.m. with 1000 *T. borreli* and given regular i.v. injections of 0.1 ml serum. The first injection was given 3 days p.i. Fish were injected with (non)immune serum, either heated for 30 min at 50 °C to inactivate complement activity, or left untreated. The serum injections were carried out on the first, third and fifth days of each week, for 4 w.p.i. Immune sera were obtained from outbred carp that had survived an infection with *T. borreli*, and the non-immune control sera were from trypanoplasm-free outbred carp. Serum pool antibody titres, determined by serial dilution with enzyme-linked immunosorbent assay (ELISA), were 10 240 in immune and 40 in non-immune carp. Parasitaemia was measured at the time of passive transfer, and mortalities were recorded daily for 5 w.p.i.

Immunization with parasite-unrelated antigen

The ability of the two homozygous carp lines (E4 and E20, n=27 each) to mount an antibody response against an antigen unrelated to the parasite (DNP-KLH) was compared with that of outbred carp (n=37). For this purpose, fish were injected i.m. with 10 μg $DNP_{494}KLH$ in Freund's incomplete adjuvant (Wiegertjes *et al.*, 1994). Pre-immune serum, and serum samples collected 2 and 3 weeks after immunization, were analyzed for DNP-specific antibodies.

ELISA

Antibody elicited in response to *T. borreli* infection, was measured using an enzyme-linked immunosorbent assay (ELISA; Jones *et al.*, 1993). Briefly, 3 x 10^4 trypanoplasms, purified from the blood of heavily infected carp, and lysed by freezing and thawing three times, were coated in carbonate buffer (pH 9) onto 96-well ELISA plates. Succeeding steps

included blocking with 3% (w/v) BSA, incubation with test sera (1:100; in triplicate), incubation with mouse monoclonal anti-carp immunoglobulin (WCI-12; 1:500), and with goat-anti-mouse-Ig-horseradish peroxidase (1:2000). Colour development was detected with *o*-phenylene-diamine and H_2O_2 in citrate-phosphate buffer (pH 5). Antibody titres were expressed as optical density (OD) readings at 492 nm.

The DNP-specific antibody response to DNP-KLH immunization was measured by ELISA, as described previously (Wiegertjes *et al.*, 1994). In short, 96-well ELISA-plates were coated with 0.10 μg ml^{-1} $DNP_{44}BSA$ in carbonate buffer. Further incubation steps were similar to the description above. Antibody titres (serial dilutions), were expressed as OD readings at 492 nm, correlating to 1:500 serum dilution (in duplicate).

Statistical analysis

Mean survival times (MST) were based on the total number of deaths at 5 w.p.i., and differences between groups were tested for significance ($P \leq 0.05$) with analysis of variance (ANOVA). Antibody titres were compared with Student's *t*-test (Statistical Analysis Systems Institute Inc., 1990).

Results

Parasite infection

All (24/24) hybrid (E20 x E6) carp died from high parasitaemia, following i.m. infection with 5 x 10^4 *T. borreli*, in clear contrast with the outbred group where only 29% of the animals died (7/24). Typically, in both groups, trypanoplasms were first detected in the blood 2 w.p.i., preceding a rapidly increasing parasitaemia. Maximum numbers of 10^8-10^9 *T. borreli* ml^{-1} were seen in susceptible fish from both groups, shortly before death at 3-4 w.p.i. (data not shown). The hybrid (E20 x E6) carp died significantly sooner (MST 22.1 days) than the susceptible outbred animals (MST 24.4 days; $P \leq 0.05$). Outbred carp showed a significant increase in antibodies specific to *T. borreli*, which started between 2 and 3 w.p.i. (Fig. 1). In contrast, antibody titres in the hybrid (E20 x E6) carp showed no increase, but declined until 3 w.p.i., followed by the death of the animals.

Despite the low dose of 100 trypanoplasms per fish, none of the homozygous carp from either line (E4, E20) survived infection (Fig. 2). In contrast, the majority (85%) of the outbred animals did survive. Again, susceptible outbred carp lived slightly longer ($P \leq 0.01$, see Table 1). The difference in time to death between the homozygous carp lines was small but significant ($P \leq 0.001$); E20 carp lived approximately 2 days longer (MST 25.1 days) than E4 carp (MST 22.8 days; Table 1).

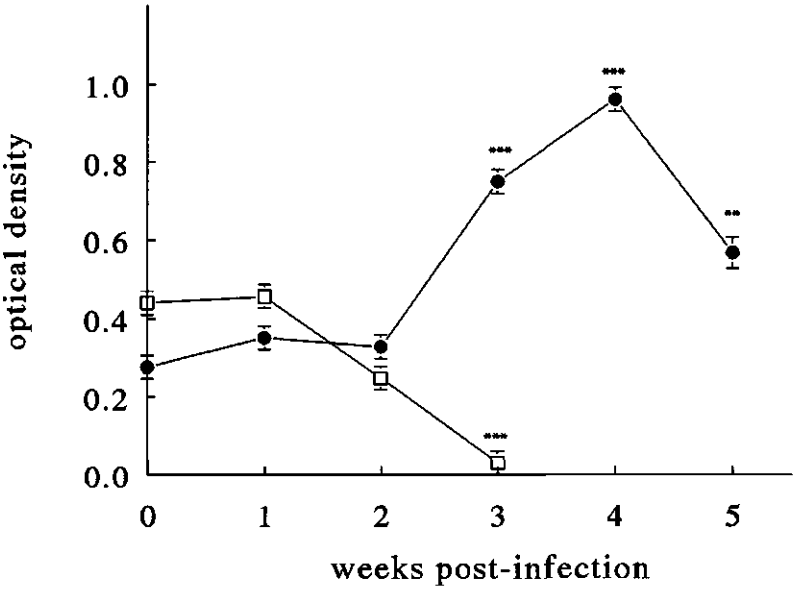


Figure 1. Antibody response (mean±SE) of hybrid E20 x E6 (□) and outbred (●) carp, i.m. infected with 5 x 10^4 *T. borreli* per fish. Each point represents the mean of n=24 (pre-immune) or two randomly assigned fish (other measurements). ** $P \leq 0.01$; *** $P \leq 0.0001$; compared with pre-immune values (t test).

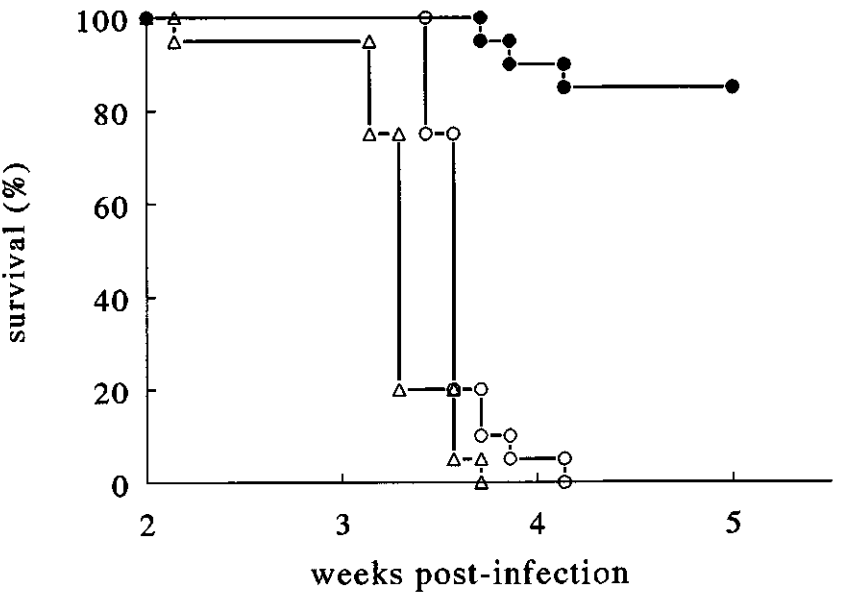


Figure 2. Mean survival of homozygous E4 (△), E20 (○) and outbred (●) carp following duplicate (2x n=10) i.m. infection with 100 *T. borreli* per fish.

Table 1. Mean survival time (MST±SE) of homozygous (E4, E20) and outbred carp after i.m. infection with 100 *Trypanoplasma borreli*.

Group[a]	MST (days)	No. of deaths/No. of infected
E4	23.8±0.2**	10/10
	21.9±0.6**	10/10
E20	25.4±0.2**	10/10
	24.9±0.1**	10/10
Outbred	26.5±0.1	2/11
	35.0	1/9

[a] in duplicate; ** $P \leq 0.01$; compared with outbred carp (ANOVA).

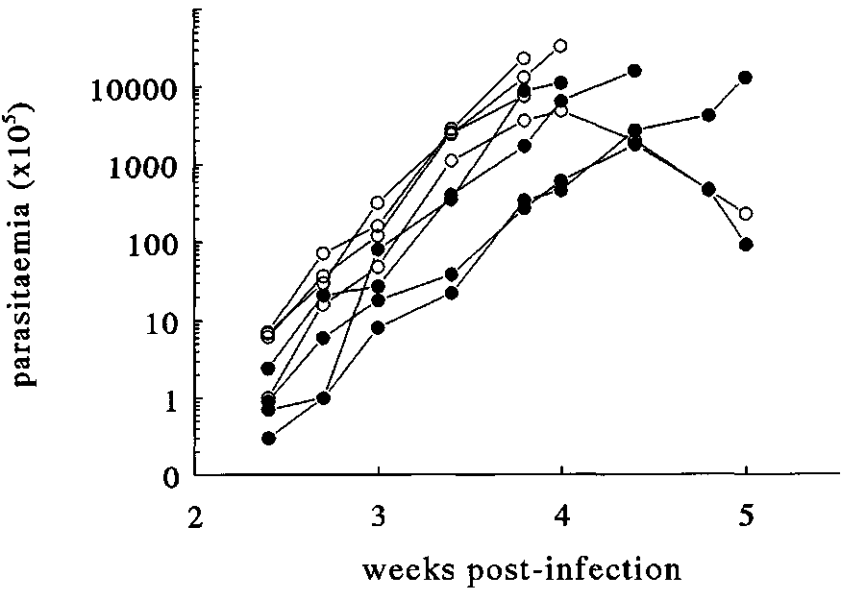


Figure 3. Parasitaemia following i.m. infection of hybrid carp (E20 x E6) with 1000 *Trypanoplasma borreli* and regular (three times per week) i.v. injections of 0.1 ml untreated immune (●; n=4) or non-immune (○; n=4) sera. Each line represents the values for one carp until the individual's death, recorded until 5 weeks post-infection.

Table 2. ELISA values expressed as optical density readings at 492 nm (mean±SE) before, and 2 and 3 weeks after immunization of homozygous (E4, E20) and outbred carp with DNP-KLH.

Group	Pre-immune	2 weeks	3 weeks	*n*
E4	0.14±0.01	0.22±0.01	0.16±0.01	27
E20	0.97±0.01	0.93±0.01	0.91±0.01	27
Outbred	0.10±0.00	0.28±0.01**	0.67±0.01***	37

** $P \leq 0.01$; *** $P \leq 0.0001$, compared with pre-immune values (*t*-test).

Passive immunization

The apparent relation between susceptibility and the absence of an antibody response, initiated an investigation into the role of specific antibody in the defence against the trypanoplasm. The passive transfer of immune serum that was left untreated, reduced the parasitaemia approximately five-fold (between 2 and 4 w.p.i.), compared with the parasitaemia in fish receiving non-immune control serum (see Fig. 3). Although the fish that received untreated immune serum generaly did not survive infection (1/4 only), the survival time of this group was somewhat prolonged (MST 33 days). The group that received untreated non-immune control serum, and also the fish that received heated (non)immune sera, all developed comparable parasitaemias and subsequently died at a MST of 27 days (data not shown).

Immune response to parasite-unrelated antigen

To exclude a possible low antibody response caused by a poor state of health of animals highly susceptible to the trypanoplasm, the homozygous carp were immunized with an antigen-complex unrelated to *T. borreli*. It was found that both lines (E4, E20) were unable to produce antibody titres higher then pre-immune levels (Table 2). This was in clear contrast with the outbred group, where increased antibody levels were detected at 2 and 3 weeks post-infection. Remarkably, high pre-immune anti-DNP antibody levels were observed in all carp from the E20 line tested for anti-DNP response.

Discussion

The use of fish for research is seriously hampered by the lack of inbred, or isogenic, lines. Hybrid F_1s between homozygous fish, obtained by gynogenesis, provide isogenic lines suitable for biological analysis of host-parasite relationship(s) in fish. In outbred carp, antibody levels and peak antibody production against *T. borreli* corresponded with elimination of the parasite, similar to the observations by Jones *et al.* (1993). Hybrid F_1 carp however, were unable to respond with an increase in antibodies against *T. borreli*, and antibody levels actually declined in these fish. The decline was probably caused by the poor health status of these animals, caused by peak parasitaemia. In fact, only recently we observed that these carp are able to survive infection and produce specific antibodies, but only, for instance, when infected with very low doses (5-10) of trypanoplasms (unpublished observations). In carp highly susceptible to infection with the trypanoplasm, the log phase growth of the flagellate may overpower the (humoral) immune response, as suggested by Burreson and Frizell (1986) for infection with a related parasite (*Trypanoplasma bullocki*) in summer flounder (*Paralichtys dentatus*). Low, or merely late, antibody responsiveness of the parasite-susceptible hybrid F_1 would explain their high mortality relative to outbred animals.

We examined the role of antibody production, in the defence of hybrid F_1 carp to *T. borreli*, by passive immunization with immune serum. Transfer of immunity, by this method, was only partial as shown by a slightly longer survival time of animals injected with untreated immune serum. Injection of heated immune serum did not have a protective effect, suggesting complement activation also plays an important role. Ahmed (1994) was able to protect carp from a lethal infection with *Trypanosoma danilewskyi*, a haemoflagellate related to *T. borreli*, by a single injection with plasma from carp that had recovered from an infection with the same parasite. The protective plasma had a higher lytic activity against *T. danilewskyi*, as detected *in vitro*, compared with control plasma. However, since complement activity was not inactivated, and no specific antibody levels were measured, the mechanisms responsible for protection remain undefined. Jones and Woo (1987) were able to protect rainbow trout (*Oncorhynchus mykiss*) against a related parasite *Cryptobia salmositica*, by passive transfer of combinations of leucocytes and immune plasma, only. They suggested that complement and lysis were important in the acute phase, while sensitization of phagocytes would be more important during the chronic stage of infection.

All homozygous carp died when infected with a dose of only 100 *T. borreli*, in clear contrast to the situation in the outbred group, in which the majority of fish (71%) survived infection. This confirms the observations of Jones *et al.* (1993) on outbred carp, in which an acute early phase of high parasitaemia was followed by elimination of the parasite. Genetic

variation among outbred individuals in the ability to mount an immune response against the parasite was suggested by extreme variability in parasitaemia. Similarly, Bower and Margolis (1984) observed genetic differences in innate susceptibility of Pacific salmon (*Oncorhynchus* spp.) to the haemoflagellate *C. salmositica*. Some salmon stocks suffered high mortalities at low exposure (100 flagellates), whereas others were resistant to high exposures of 10^6 flagellates.

In homozygous carp lines immunized with a hapten-carrier antigen (DNP-KLH), unrelated to the trypanoplasm, it was found that the carp were unable to produce increased anti-DNP antibody levels, which also argues for a low antibody responsiveness of these genotypes. No more E20 x E6 F_1 animals, that responded with a decline in antibody levels upon infection with *T. borreli*, were available for DNP-KLH immunization. The inability to respond to DNP-KLH however, in contrast with outbred animals, has been confirmed in several genetically related hybrid F_1s (E20 x E5, E4 x E5, E4 x E6; unpublished observations). The failure of all carp within the homozygous lines to (rapidly) respond to two unrelated antigens (*T. borreli* and DNP-KLH) suggests that antibody production in these lines is under genetic control.

To answer questions relating to the immunological mechanisms behind susceptibility to *T. borreli* infection, it would be rewarding to select for immune characteristics which may have an influence on host resistance, as has been done with the Biozzi mouse lines selected for high and low antibody response (Biozzi *et al.*, 1979). These mouse lines were shown to be resistant or susceptible to *Trypanosoma cruzi* infection, respectively, and resistance could be passed on to the susceptible line by passive immunization with immune serum (Kierszenbaum and Howard, 1976). We have started a divergent selection of carp, using artificial gynogenesis to breed for antibody production to DNP-KLH (Wiegertjes *et al.*, 1994). The aim is to obtain high and low antibody responder isogenic carp lines, which could further facilitate investigations of a genetic regulation of host-parasite relationship(s) in fish.

Acknowledgements

T. borreli was kindly provided by Dr. D. Steinhagen, Hannover, Germany. This research was financially supported by the Netherlands Technology Foundation (SLW), and was coordinated by the Life Sciences Foundation (SLW). The authors wish to thank A. van den Broek, G.D. Daniels, P. Heinen, A. Luttikholt and N.M.S. Santos for their technical assistance. The critical reading of the manuscript by Drs R.J.M. Stet and S.R.M. Jones is highly appreciated.

References

Ahmed, M.S. 1994. Trypanosomiasis in common carp (*Cyprinus carpio* L.). PhD thesis, Catholic University Leuven, Belgium.

Biozzi, G., Mouton, D., Sant'Anna, O.A., Passos, H.C., Gennari, M., Reis, M.H., Ferreira, V.C.A., Heumann, A.M., Bouthillier, Y., Ibanez, O.M., Stiffel, C. and Siqueira, M. 1979. Genetics of immunoresponsiveness to natural antigens in the mouse. Curr. Top. Microbiol. Immunol. 85, 31-98.

Bower, S.M. and Margolis, L. 1984. Detection of infection and susceptibility of different Pacific salmon stocks (*Oncorhynchus* spp.) to the haemoflagellate *Cryptobia salmositica*. J. Parasitol. 70, 273-278.

Burreson, E.M. and Frizell, L.J. 1986. The seasonal antibody response in juvenile summer flounder (*Paralichtys dentatus*) to the haemoflagellate *Trypanoplasma bullocki*. Vet. Immunol. Immunopathol. 12, 395-402.

Festing, M.F.W. and Blackwell, J.M. 1988. Determination of mode of inheritance of host response. In: Genetics of resistance to bacterial and parasitic infection (eds: Wakelin, D.M. and Blackwell, J.M.). Taylor & Francis, New York, pp. 21-61.

Han, H-S., Taniguchi, N. and Tsujimura, A. 1991. Production of clonal ayu by chromosome manipulation and confirmation by isozyme marker and tissue grafting. Nippon Suisan Gakkaishi 57, 825-832.

Hörstgen-Schwark, G. 1993. Production of homozygous diploid zebra fish (*Brachydanio rerio*). Aquaculture 112, 25-37.

Jones, S.R.M., Palmen, M. and van Muiswinkel, W.B. 1993. Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. Vet. Immunol. Immunopathol. 36, 369-378.

Jones, S.R.M. and Woo, P.T.K. 1987. The immune response of rainbow trout, *Salmo gairdneri* Richardson, to the haemoflagellate, *Cryptobia salmositica* Katz, 1951. J. Fish Dis. 10, 395-402.

Kierszenbaum, F. and Howard, J.G. 1976. Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. J. Immunol. 116, 1208-1211.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.) II. The production of homozygous gynogenetic clones and F_1 hybrids. Aquaculture 92, 127-142.

Lom, J. 1979. Biology of the trypanosomes and trypanoplasms of fish. In: Biology of the kinetoplastida (eds: Lumsden, W.H.R. and Evans, D.A.). Academic Press, London, pp. 269-337.

Naruse, K., Ijiri, K., Shima, A. and Egami, N. 1985. The production of cloned fish in the medaka (*Oryzias latipes*). J. Exp. Zool. 236, 335-341.

SAS Institute Inc. 1990. SAS User's Guide: Statistics. Version 6, 4th edn, Vol. 2. SAS Institute Inc., Cary, NC.

Steinhagen, D., Kruse, P. and Körting, W. 1989. The parasitaemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). J. Parasitol. 75, 685-689.

Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291, 293-296.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1994. Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. Anim. Genet. 25, 251-257.

Chapter 5

Investigations into the ubiquitous nature of high or low immune responsiveness after divergent selection for antibody production in common carp (*Cyprinus carpio* L.)

Geert F. Wiegertjes, René J. M. Stet and Willem B. van Muiswinkel

Department of Experimental Animal Morphology and Cell Biology
Wageningen Agricultural University
P.O. Box 338, 6700 AH Wageningen, The Netherlands

Published in: **Veterinary Immunology and Immunopathology 48, ...-... (1995)**

Summary

This paper reports on the selection of individual carp with a high or low antibody response, in combination with reproduction by gynogenesis, in order to develop well-characterized inbred carp lines consisting of practically unlimited numbers of carp with the same genotype. Two homozygous progenies, previously characterized as having a high or low immune responsive to dinitrophenyl keyholelimpet haemocyanin (DNP-KLH), were immunized with either a T cell-dependent (DNP-human serum albumin (DNP-HSA)) or T cell-independent (trinitrophenyl lipopolysaccharide (TNP-LPS)) hapten-carrier complex. In comparison with the antibody response after DNP-KLH immunization, the response to DNP-HSA was observed to be highly variable and did not differ between the divergently selected progenies. This suggests that the divergent selection for antibody production to DNP-KLH has been carrier-specific. Immunization with T-independent TNP-LPS induced a very rapid response which differed between the high and low responders, and likely measured changes in the DNP-specific precursor pool of B cells caused by the selection. A number of selected individuals with a high immune response to DNP-KLH were infected with *Trypanoplasma borreli*, a haemoflagellate parasite of carp, to examine a possible relationship between the increase in immune responsiveness and disease resistance, but no change could be detected. However, individual homozygous carp were able to escape inbreeding depression and survive the infection. Such carp would be likely candidates for gynogenetic reproduction to obtain viable inbred carp lines.

Key words: *Cyprinus carpio* L., immune responsiveness, disease resistance

Introduction

Despite the awareness that "inbred lines have led to rapid progress and became indispensable in virtually every branch of the biomedical sciences" (Klein, 1986), most of the immunological research in fish is still done on outbred animals. Streisinger *et al.* (1981) reported the use of artificial gynogenesis for the rapid production of fully inbred fish lines, and similar reproduction methods have now been developed for a number of fish species. However, technical problems have apparently limited the availability of large numbers of identical fish for immunological studies, and only a few isolated reports have been published. For example, exchange of allografts between inbred fish lines reduced the number of unassigned histocompatibility loci to one or two major loci (reviewed in Stet and Egberts, 1991). Preliminary studies in gynogenetic carp invariably showed low autologous responses in mixed leucocyte reactions and a reduced variation in the percentage immunoglobulin-positive peripheral blood lymphocytes (G.F. Wiegertjes *et al.*, unpublished data, 1991).

The selective reproduction of individuals with defined immunological phenotypes, using artificial gynogenesis, was initiated in order to develop well-characterized inbred carp lines. Similarly to studies on, for example, mice (Biozzi *et al.*, 1979) and chickens (van der Zijpp and Nieuwland, 1986), we divergently selected animals with a high or a low antibody response.

In contrast to the use of sheep red blood cells as multideterminant antigen, we chose dinitrophenyl keyholelimpet haemocyanin (DNP-KLH) as selection antigen (Wiegertjes *et al.*, 1994). The divergent selection for antibody production against the hapten (DNP) will probably result in inbred fish lines with a genetic difference in immune regulation. The limited structural heterogeneity of the hapten could be a key factor in unmasking major histocompatibility complex (Mhc) associated immune response (Ir) genes controlling the response of helper T cells (Klein, 1986).

The use of gynogenesis for the production of inbred carp (*Cyprinus carpio* L.) lines requires a procedure that involves two generations of 1.5-2 years each. The first generation is obtained after activation of eggs with sperm, irradiated with UV-light, followed by temperature-shock inhibition of the first mitotic division. The resulting homozygous fish express female alleles only, but are all expected to be different. These females can be reproduced by a similar methodology to obtain inbred lines that consist of practically unlimited numbers of homozygous animals with the same genotype (Komen *et al.*, 1991).

The genetic control of the antibody response in fish has received little attention, despite a clear interest of fish breeders in indirect markers that can be used to select high responder animals with an increased resistance to disease (reviewed by Chevassus and Dorson, 1990). Recently, Strønsheim *et al.* (1994a, b) described genetic variation in the humoral immune response of Atlantic salmon (*Salmo salar*) to multideterminant antigens (*Aeromonas salmonicida* A-layer; *Vibrio anguillarum* O-antigen). We have shown that the average antibody response of gynogenetic progeny obtained from parents that were divergently selected for antibody production to DNP-KLH, was consistent with the high or low responder type of the parent (Wiegertjes *et al.*, 1994). To extend these findings, we used two such progenies, previously characterized for their high or low immune responsiveness to DNP-KLH, and immunized these carp with either a T cell-dependent or T cell-independent hapten-carrier complex. Subsequently, individual high responding carp were infected with *Trypanoplasma borreli*, a haemoflagellate parasite of carp, to examine a possible relationship between the increase in immune responsiveness and a change in disease resistance.

Materials and methods

Divergent selection

A divergent selection for primary antibody production against DNP-KLH was initiated using the female offspring of an F_1 hybrid cross between a male common carp (*Cyprinus carpio* L.) of Polish (R3) and a female common carp of Hungarian (R8) origin. Six animals from this (R3 x R8) base population were selected for their high (Nos 17, 60, 69) or low (Nos 51, 60, 85) antibody response, and gynogenetically reproduced using inhibition of the first mitotic division according to Komen *et al.* (1991). The resulting homozygous progenies were randomly sampled at the age of 6 months, and the differences in immune responsiveness between the high and low responder groups suggested genetic control of the antibody production to DNP-KLH (Wiegertjes *et al.*, 1994). Two homozygous progenies (from Nos 17, 60), characterized as high and low responders to DNP-KLH respectively, were used as the experimental animals in the present investigation. These carp will henceforth be denoted as progeny No. 17 and progeny No. 60.

Husbandry

Carp were grown at 25 ± 2 °C in recirculated, filtered and UV-treated water. After initial feeding on *Artemia nauplii* for a period of 3 weeks following yolk-sac absorption, the feeding regime was switched to a daily ration of pelleted trout food (Trouvit, Trouw and Co., Putten, The Netherlands) of 25 g kg^{-1} metabolic weight. The two progenies were reared in separate 140 l aquaria of the same recirculation system until 1 month before immunization, when both progenies were randomly divided over six 140 l aquaria of the same system. To allow the fish to acclimate, approximately 3 months before the infection experiment with *T. borreli*, carp were transported to a flow-through system of separate 120 l aquaria at 20 ± 2 °C.

Before immunization, or bleeding by caudal venipuncture, fish were anaesthetized in 0.02% (w/v) tricaïne methane sulphonate (Crescent Research Chemicals, Phoenix, AZ) solution. Blood was allowed to clot at room temperature for 1 h to obtain serum samples for antibody measurements, which were stored at -20 °C.

Immunization with hapten-carrier complex

Carp of 14 months old and weighing 325 ± 91 g from progeny No. 17 (n = 59; high responders) or from progeny No. 60 (n = 58; low responders) were immunized with a T cell-dependent (DNP-KLH, DNP-human serum albumin (DNP-HSA)) or T cell-independent (trinitrophenyl lipopolysaccharide (TNP-LPS)) hapten-carrier complex (n=10 per antigen, in duplicate). The fish were injected intramuscularly (i.m.) with 10 μg $DNP_{494}KLH$ (Calbiochem, La Jolla, CA), with 10 μg $DNP_{35}HSA$ (Calbiochem) or with 10 μg TNP-LPS (*Escherichia coli*

Serotype 0111:B4; Sigma Chemical Co., St. Louis, MO) in Freund's incomplete adjuvant. The immunization procedure, and the enzyme-linked immunosorbent assay (ELISA) used to detect serum antibodies, have been described in detail previously (Wiegertjes *et al.*, 1994). Briefly, 96-well ELISA plates were coated overnight at 37 °C with 0.10 μg ml^{-1} $DNP_{44}BSA$ (DNP-bovine serum albumin, Calbiochem) to detect DNP-specific antibodies in serum samples (1:100, in triplicate) collected before immunization, and 12, 21 and 28 days after immunization. Then, plates were incubated with WCI-12, a mouse monoclonal antibody against carp immunoglobulin (Secombes *et al.*, 1983), and subsequently with goat anti-mouse horseradish peroxidase (GAM-HRP, BioRad, Richmond, CA, USA). Orthophenylenediamine substrate incubation was stopped with sulphuric acid, and colour development was detected at 492 nm with a spectrophotometer.

Statistical significance ($P<0.05$) of differences in DNP-specific antibody production (analysis of variance) was tested with standard procedures (Statistical Analysis Systems Institute Inc., 1990).

Parasite infection

Carp of progeny No. 17, identified as high responders to DNP-KLH ($n=4$ animals with the highest response), were infected with *T. borreli* 3 months after measurement of the antibody response (age 18 months). The control group (progeny No. 17) consisted of carp taken after measurement of the antibody response to DNP-HSA ($n=10$; random sample). The average weight of the carp was 780 ± 147 g at the time of immunization.

Trypanoplasma borreli (Kinetoplastida: Cryptobiidae) was cloned and characterized by Steinhagen *et al.* (1989). Carp were i.m. infected with 1000 haemoflagellates. Parasitaemia was monitored by microscopical examination of heparinized blood samples collected at 2 and 3 weeks after infection. Parasites were counted using a Bürker counting chamber. Dead fish were recorded and removed daily for 6 weeks.

In order that the sampling procedure should not influence survival time, blood samples to measure serum antibody elicited to *T. borreli* were taken only after 5 weeks following infection. Specific antibody production was measured with an ELISA previously described in detail by Jones *et al.* (1993). Briefly, 3×10^4 trypanoplasms, isolated from the blood of heavily infected carp, and lysed by freezing and thawing three times, were coated onto ELISA plates in coating buffer. The subsequent steps were similar to the ELISA described above.

Results

Hapten-carrier immunization

At 6 months of age, fish of progeny No. 17 were characterized as high responders, and fish of progeny No. 60 as low responders to DNP-KLH. To investigate the ubiquitous nature of the immunogenetic change in these fish, they were immunized with a T cell-dependent (DNP-KLH, DNP-HSA) or T cell-independent (TNP-LPS) hapten-carrier complex. Analysis of variance suggested the absence of significant tank effects and allowed the duplicate groups to be pooled (n=19-20 per antigen). DNP-KLH immunization was again included in the experimental set-up and, as expected, the antibody titres were higher in progeny No. 17 than in fish of progeny No. 60, although the difference was smaller than detected at 6 months (Table 1).

The various hapten-carrier complexes induced clearly different antibody responses, which sometimes differed between the high and low responder progenies (Fig. 1). Immunization with T-dependent antigen DNP-HSA, which, compared with the selection antigen DNP-KLH, consisted of the same hapten coupled to a different carrier, resulted in an average antibody response which was significantly ($P < 0.05$) lower than the response to DNP-KLH, at all measurements after immunization. In addition, the divergently selected difference between the high and low responder group was undetectable ($P > 0.90$) after DNP-HSA immunization.

Characteristic of the antibody response to T-independent antigen TNP-LPS was that the increase in antibody response was more rapid than seen after immunization with the T-dependent hapten-carrier complexes, as shown by a significantly higher ($P < 0.01$) response at 12 days after immunization. The antibody production to TNP-LPS increased only slightly after 12 days and remained significantly ($P < 0.001$) lower at 21 and 28 days than the antibody production to DNP-KLH (but not to DNP-HSA). In contrast to our inability to detect a difference in antibody production to DNP-HSA between the high and low responder progenies, immunization with T-independent TNP-LPS induced a significantly different antibody response, both at 21 and at 28 days ($P < 0.01$) after immunization.

To gain insight into the distribution of the variation within homozygous groups, each individual antibody response was plotted per antigen complex, per day (Fig. 2). The distribution of the variation in antibody responses, within the high (No. 17) and low (No. 60) responder progenies was highly comparable, and only the response of progeny No. 17 is shown. The progenies were generally characterized by a large variation between the homozygous individuals. The lowest variation was seen in the group immunized with the selection antigen DNP-KLH (Fig. 2a), as demonstrated by the relatively low coefficients of variation (CV) in this group (e.g. CV = 15.1 at 21 days).

Table 1. Antibody response[a] of the homozygous progenies, gynogenetically produced from high and low responder carp selected from the R3 x R8 base population. The data obtained after immunization at 6 months of age are included for comparison.

Group	Age (months)	*n*	Mean (± SE) optical density			
			pre-immune	12 days	21 days	28 days
High responder progeny						
No. 17	6	10	0.39±0.07	0.30±0.05	1.04±0.13	1.56±0.16**
	14	20	0.20±0.04	0.45±0.10	1.65±0.06	1.89±0.04*
Low responder progeny						
No. 60	6	14	0.48±0.07	0.45±0.04	1.00±0.09	1.27±0.10
	14	19	0.18±0.03	0.43±0.05	1.59±0.06	1.76±0.06

[a] Immunizations were with DNP-KLH. Serum antibodies (1:100) were measured by ELISA, before, and 12, 21 and 28 days after immunization. ** $P < 0.05$, compared with low responders at 6 months; * $P < 0.10$, compared with low responders at 14 months.

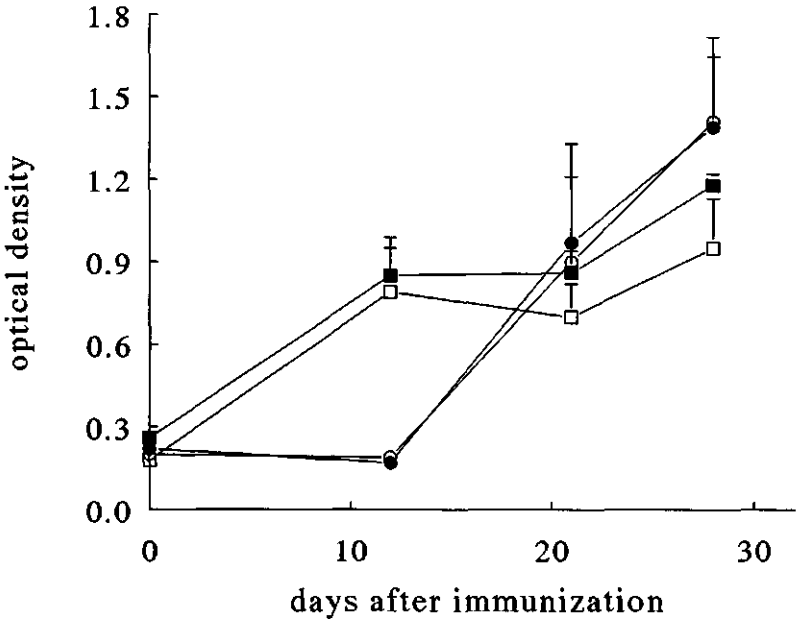


Figure 1. DNP-specific antibodies (optical density) in response to immunization with T cell-dependent DNP-HSA (circles) or with T cell-independent TNP-LPS (squares). Carp were previously characterized as high (progeny No. 17, closed symbols) or low (No. 60, open symbols) responders to DNP-KLH. The antibody response was measured before, and 12, 21 and 28 days after immunization.

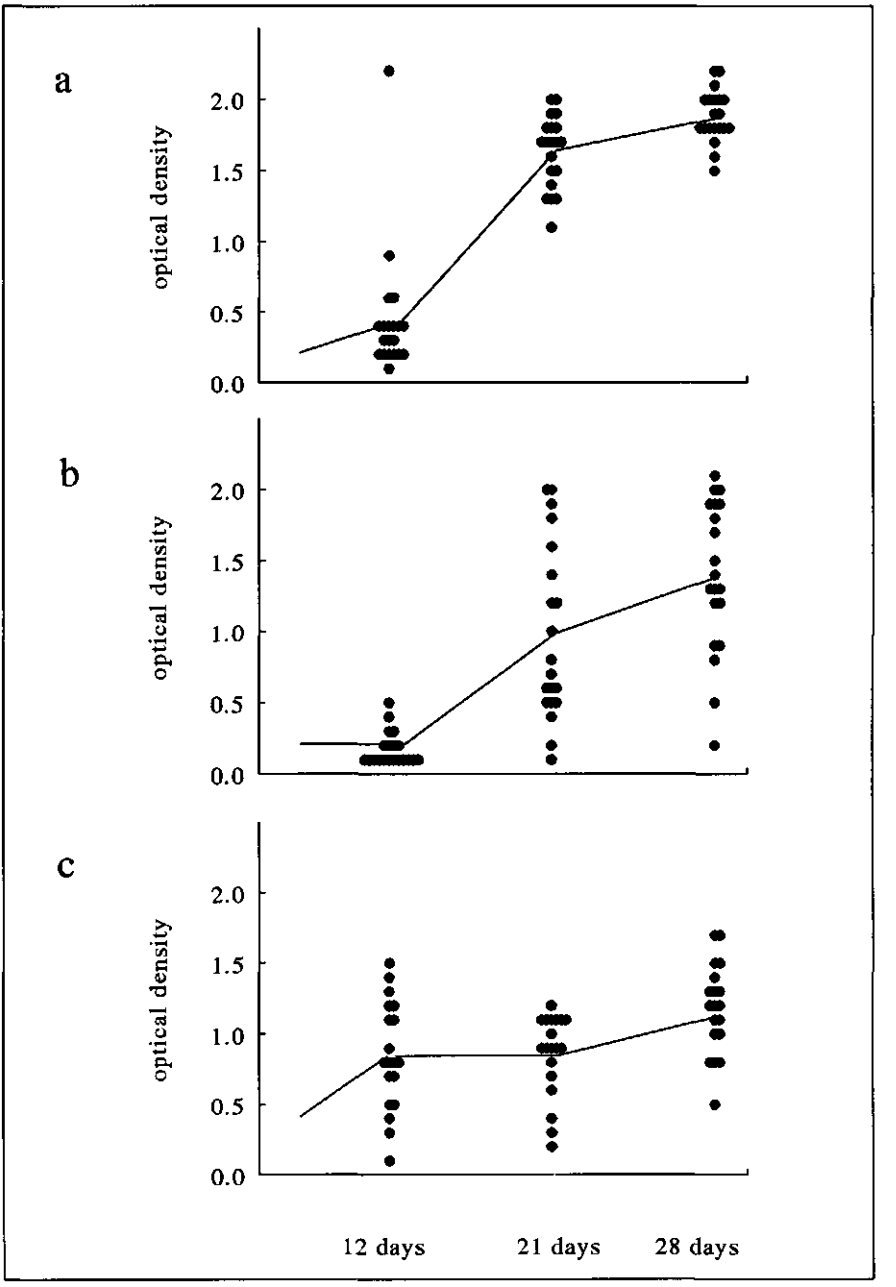


Figure 2. Distribution of the variation, within progeny No. 17 (high responders), in DNP-specific antibodies (optical density) in response to immunization with T cell-dependent DNP-KLH (**a**), DNP-HSA (**b**) or with T cell-independent TNP-LPS (**c**). The antibody response at 12, 21 and 28 days after immunization is shown.

Immunization with DNP-HSA however, did not induce the uniform response described above (Fig. 2b). Some animals reacted with a high antibody response, and a considerable number of animals reacted with a low, or late, antibody response. These differences in response caused an extreme variation in antibody titres (e.g. CV = 63.9 at 21 days). In general, the variation in antibody production after immunization with TNP-LPS (Fig. 2c) was intermediate (e.g. CV = 33.7 at 21 days).

Parasite infection

Progeny No. 17 were characterized as high responders to DNP-KLH, based upon the average response of the group, both at 6 and 14 months of age (see Table 1). This means that, within the progeny, an increased number of animals will show high immune responsiveness. However, as shown in Fig. 2, the variation within homozygous progenies is generally high. To use truly high responders within progeny No. 17, only individual carp with the highest antibody production to the selection antigen (DNP-KLH) were used to detect a possible relationship with resistance (or susceptibility) to *T. borreli*. The control group consisted of fish from the same progeny taken after DNP-HSA immunization, which, based upon the results shown in Fig. 1 and 2, represent a random group with the same genetic background.

The establishment of infection was confirmed at 2 weeks, for each individual, by a positive but low parasitaemia ((1-5) x 10^4 *T. borreli* ml^{-1} blood; data not shown). At 3 weeks after infection, the variation in parasitaemia, between the homozygous carp, was fifty-fold (1 x 10^6-5 x 10^7) and suggested a variation in susceptibility to the parasite. To investigate the use of these parasitaemia values as parameter for resistance to the parasite, first, the relation between parasitaemia at 3 weeks and survival time was examined (for all infected carp). A high parasitaemia generally correlated with a shortened survival time (Fig. 3), which indeed suggested that the parasitaemia at 3 weeks could be used to detect a possible change in disease resistance in the selected group.

No differences in resistance to the parasite, measured as parasitaemia, survival time or as mortality, could be detected between the high responders and the control group (Table 2). The majority of the carp (12/14) died as a result of the apparently high virulence of the parasite, which minimized the variation in resistance between the experimental groups, and thus reduced the sensitivity of the assay. Some homozygous individuals however, were able to survive the infection, including one animal with a high response to DNP-KLH, and one carp of the control group. These fish were bled for antibodies against the trypanoplasm, and showed increased antibody titres (1:1280-1:2560) compared with non-infected animals (1:40-1:80), from 5 weeks onwards, and no detectable parasitaemia at 8 weeks.

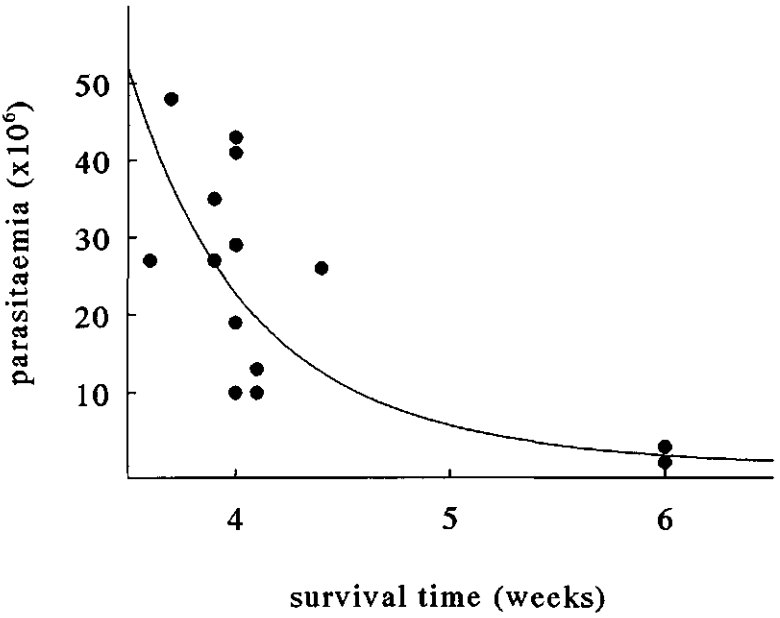


Figure 3. Relation between parasitaemia (haemoflagellates ml^{-1} blood) detected at 3 weeks after i.m. infection with 1000 *T. borreli*, and survival time.

Table 2. Resistance to infection with *Trypanoplasma borreli* of high responder carp and a control group of the same genetic background (progeny No. 17). Resistance was measured as parasitaemia (*T. borreli* ml^{-1} blood) 3 weeks after infection, survival time, and mortality (number of dead/infected fish). Data are presented as individual values.

Immune responsiveness	Resistance to *T. borreli*		
	Parasitaemia ($x10^6$)	Survival time (days)	Mortality
High	1 19 27 35	27 27 28 >42	3/4
Unknown (control)	2 10 10 13 26 27 29 41 43 48	25 26 28 28 28 28 29 29 31 >42	9/10

Discussion

We initiated a divergent selection for antibody production in carp, based upon the reaction to a hapten-carrier complex (DNP-KLH), and demonstrated a genetic control of the immune response (Wiegertjes *et al.*, 1994). The use of well-defined antigenic determinants to select for immune responsiveness, increases the detection level of a genetic determination, but may not have a causative link with resistance or susceptibility to disease. To test the ubiquitous nature of the high and low immune responsiveness after selection, high and low responder carp were immunized with other hapten-carrier complexes (DNP-HSA, TNP-LPS). Subsequently, high responder carp were infected with a pathogenic organism (*Trypanoplasma borreli*) to detect a possible link with resistance to disease.

The carp that were used in the present study were previously characterized as high or low responders to DNP-KLH at the age of 6 months (Wiegertjes *et al.*, 1994). These fish were 14 months old at immunization, and in agreement with the previous findings, DNP-KLH again induced a difference in antibody production. The selection difference seemed to decrease slightly with age, in contrast to recent measurements of the difference between comparable high and low responder groups (Nos 69 and 85, respectively), showing larger differences at a later age (G.F. Wiegertjes *et al.*, unpublished data, 1994). The variation in antibody response after immunization with DNP-KLH was relatively low, but was highly variable after immunization with DNP-HSA. The large variation in response to T cell-dependent DNP-HSA suggests that the homozygous progenies represent populations in which no prior selection occurred regarding immune responsiveness to the HSA carrier. This is supported by the observation that the high and the low responder groups showed the same magnitude of response after immunization with DNP-HSA. T cell-dependent hapten-carrier complexes, such as DNP-KLH and DNP-HSA, are assumed to stimulate both carrier-specific T cells and hapten-specific B cells. The results thus seem to suggest a KLH (carrier)-specific immunogenetic change in the selected groups.

Immunization with a T-independent complex (TNP-LPS) caused a very rapid increase in antibody production. The efficacy of TNP-LPS in eliciting an antibody response has been reported previously for other fish species (*Carassius auratus*, Desvaux and Charlemagne, 1981; *Oncorhynchus mykiss*, Arkoosh and Kaattari, 1991). T-independent complexes are assumed to stimulate B cells without the need for T cell cooperation, which may explain the efficacy of the response. Similar to the observed differences in response to DNP-KLH, immunization with TNP-LPS also induced a difference in antibody production between the high and low responder progenies. In the present experiments, the antibody production after TNP-LPS immunization may be a measure for the size of the nitrophenyl-sensitive precursor pool of B cells, which may have changed owing to selection. Arkoosh and Kaattari (1991), in their study on memory formati-

on, suggested that the secondary response in fish may be a simple expansion of B cell precursors responsive to the antigen. Although our selection procedure was based on primary immune responses, limiting dilution assays of leucocytes taken from organs of immunized carp may answer questions regarding similar mechanisms in the selected lines.

The carrier-specific difference in the response to DNP-KLH and DNP-HSA may be controlled by Mhc-associated immune response (Ir) genes, since in mammals, and most likely in teleost fish also (Vallejo *et al.*, 1992), T cell dependent antibody production is under control of the Mhc. Although the T cell dependent response to all polypeptide antigens is under Ir gene control, the analysis of the antibody response to individual determinants (such as DNP) might circumvent the masking of Ir gene control for more complex antigens (Klein, 1986). In addition, the possibility to analyze sequence polymorphism in the peptide binding region of *MhcCyca-DAB* class II molecules (Ono *et al.*, 1993), may identify Mhc class II haplotypes with an increased incidence in high or low responder groups.

The possible relationship between high immune responsiveness after selection and a change in disease resistance was investigated by testing for a correlation of the hapten-specific antibody response with resistance to the pathogen *T. borreli*. In this disease model, peak antibody response generally coincides with a decline and eventual absence of parasitaemia, resulting in the survival of the majority of outbred carp (Jones *et al.*, 1993). In addition, we recently observed a correlation between (lack of) antibody response to DNP-KLH and (lack of) antibody response to *T. borreli* (Wiegertjes *et al.*, 1995). Although the exact nature of the immune mechanism responsible for eliminating the parasite has remained unclear, the observations suggest an important role for specific antibody production. In the present study, there was no indication that the high immune responsiveness obtained after selection for antibody production was correlated with a change in disease resistance. The carrier specificity for the selection outcome suggested above, might explain the lack of correlation. In addition, a high virulence of the parasite would explain the limited variation in resistance between individual carp. The number of injected parasites (1000) however, was lower than in previous trials (5×10^4) where the majority of (outbred) carp survived infection (Wiegertjes *et al.*, 1995). Probably, the homozygous nature of the carp caused an increased sensitivity to the flagellate owing to inbreeding depression caused by homozygous genotypes negatively influencing the resistance. Also, the large variation generally observed in homozygous groups reduces the significance of differences. However, some homozygous individuals survived the infection with *T. borreli*.

Divergent selection for antibody production to sheep erythrocytes in mice generally resulted in multispecific changes in the contribution of the regulatory role of the macrophage (Biozzi *et al.*, 1984). The selection difference could be demonstrated by differences in the antibody response to several unrelated antigens, as well as haemoflagellate pathogens such as *Trypanosoma*

cruzi (Kierszenbaum and Howard, 1976). Divergent selection for antibody production to sheep erythrocytes in chickens, as initiated by van der Zijpp and Nieuwland (1986), did not result in differences in phagocytic activity between the selected lines (Kreukniet *et al.*, 1995). These seemingly contradictory findings suggest that the immunological consequences of selection for immune responsiveness are sometimes difficult to predict and that the nature of the immunological changes can be best described afterwards. The present study is intended to be a first investigation into the ubiquitous nature of the changes that occurred during our selection procedure. The use of a hapten-carrier as selection antigen, the possibilities to characterize the Mhc, and the development of carp lines without genetic variation, may allow us to study cell cooperation *in vitro*, similar to the early studies in *Xenopus* where genetic (Mhc) control of collaboration between carrier-primed T cells and hapten-primed B cells was studied in gynogenetic, Mhc-defined inbred lines (Bernard *et al.*, 1981). Although we observed no correlation between high immune responsiveness after selection, and a change in resistance to disease, we have shown that individual homozygous carp can apparently survive an otherwise lethal infection with *T. borreli*. Such animals will be likely candidates for gynogenetic reproduction to obtain viable high and low responder inbred carp lines for experimental research.

Acknowledgements

T. borreli was kindly provided by Dr. D. Steinhagen, Hannover and the R3 and R8 carp by the Fish Culture Experimental Station Golysz. This research was financially supported by the Netherlands Technology Foundation (SLW), and coordinated by the Life Sciences Foundation (SLW). The authors wish to thank A. Groeneveld and A. Hutten for technical assistance.

References

Arkoosh, M.R. and Kaattari, S.L. 1991. Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*).I. An immunochemical and cellular analysis of the B cell response. Dev. Comp. Immunol. 15, 279-293.

Bernard, C.C.A., Bordmann, G., Blomberg, B. and Du Pasquier, L. 1981. Genetic control of T helper cell function in the clawed toad *Xenopus laevis*. Eur. J. Immunol. 11, 151-155.

Biozzi, G., Mouton, D., Sant'Anna, O.A., Passos, H.C., Gennari, M., Reis, M.H., Ferreira, V.C.A., Heumann, A.M., Bouthillier, Y., Ibanez, O.M., Stiffel, C. and Siqueira, M. 1979. Genetics of immunoresponsiveness to natural antigens in the mouse. Curr. Top. Microbiol. Immunol. 85, 31-98.

Biozzi, G., Mouton, D., Stiffel, C. and Bouthillier, Y. 1984. A major role of the macrophage in quantitative genetic regulation of immunoresponsiveness and anti-infectious immunity. Advances in Immunol. 36, 189-234.

Chevassus, B. and Dorson, M. 1990. Genetics of resistance to disease in fishes. Aquaculture 85, 83-107.

Desvaux, F.-X. and Charlemagne, J. 1981. The goldfish immune response. I. Characterization of the humoral response to particulate antigens. Immunology 43, 755-762.

Jones, S.R.M., Palmen, M. and van Muiswinkel, W.B. 1993. Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. Vet. Immunol. Immunopathol. 36, 369-378.

Kierszenbaum, F. and Howard, J.G. 1976. Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. J. Immunol. 116, 1208-1211.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley & Sons, New York.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman, E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.) II. The production of homozygous gynogenetic clones and F_1 hybrids. Aquaculture 92, 127-142.

Kreukniet, M.B., Nieuwland, M.G.B. and van der Zijpp, A.J. 1995. Phagocytic activity of two lines of chicken divergently selected for antibody production. Vet. Immunol. Immunopathol. 44, 377-387.

Ono, H., O'hUigin C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. 1993. New β chain-encoding Mhc class II genes in carp. Immunogenetics 38, 146-149.

SAS Institute Inc. 1990. SAS User's Guide: Statistics. Version 6, 4th edn, Vol. 2. SAS Institute Inc., Cary, NC.

Secombes, C.J., van Groningen, J.J.M. and Egberts, E. 1983. Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immunohistochemical studies. Immunology 48, 165-175.

Steinhagen, D., Kruse, P. and Körting, W. 1989. The parasitaemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). J. Parasitol. 75, 685-689.

Stet, R.J.M. and Egberts, E. 1991. The histocompatibility system in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. Fish Shellf. Immunol. 1, 1-16.

Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291, 293-296.

Strønsheim, A., Eide, D.M., Fjalestad, K.T., Larsen, H.J.S. and Røed, K.H. 1994a. Genetic variation in the humoral immune response in Atlantic salmon (*Salmo salar*) against *Aeromonas salmonicida* A-layer. Vet. Immunol. Immunopathol. 41, 341-352.

Strønsheim, A., Eide, D.M., Hofgaard, P.O., Larsen, H.J.S., Refstie, T. and Røed, K.H. 1994b. Genetic variation in the humoral immune response against *Vibrio salmonicida* and in antibody titre against *Vibrio anguillarum* and total IgM in Atlantic salmon (*Salmo salar*). Vet. Immunol. Immunopathol. 44, 85-95.

Vallejo, A.N., Miller, N.W. and Clem, L.W. 1992. Antigen processing and presentation in teleost immune responses. Ann. Rev. Fish Dis. 2, 73-89.

Wiegertjes, G.F., Groeneveld, A. and van Muiswinkel, W.B. 1995. Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). Vet. Immunol. Immunopathol. 47, 153-161.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1994. Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. Anim. Genet. 25, 251-257.

Zijpp van der, A.J. and Nieuwland, M.G.B. 1986. Immunological characterisation of lines selected for high and low antibody production. In: Proceedings of the 7th European Poultry Conference, Paris, pp. 211-215.

Chapter 6

Investigations into the immune responsiveness of F_1 hybrids of homozygous carp (*Cyprinus carpio* L.) selected for high or low antibody production: indications for immune response gene control

Geert F. Wiegertjes, René J. M. Stet, Augustinus B.J. Bongers[#], Paul Voorthuis, Behrouz Zandieh Doulabi[#], Adrie Groeneveld and Willem B. van Muiswinkel

Department of Experimental Animal Morphology and Cell Biology
[#] Department of Fish Culture and Fisheries
Wageningen Agricultural University
P.O. Box 338, 6700 AH Wageningen, The Netherlands

Summary

Antibody production to dinitrophenyl-keyholelimpet haemocyanin (DNP-KLH) served as the immune parameter to divergently select carp (*Cyprinus carpio* L.) with a genetically determined high or low immune responsiveness. The outcome of the selection is described by an examination of the antibody response to DNP-KLH, and other hapten-carrier complexes, in F_1 hybrids of high- or low-responder homozygous carp. It was found that, after immunization with DNP-KLH, immune sera from isogenic high-responder lines could be 1000-fold diluted, compared with immune sera from low responder carp, which could be approximately 100-fold diluted, to obtain identical values in an ELISA. This confirmed previous observations for a genetic control of the antibody response to DNP-KLH. In general, the magnitude of antibody production to T cell-independent hapten-carrier antigen (TNP-lipopolysaccharide) corresponded to the magnitude of immune response to DNP-KLH. In contrast, immunization with T cell-dependent DNP-human serum albumin induced some high-responder carp lines to become low responders. Crosses between high- and low-responder carp suggested that high responsiveness to DNP-KLH was inherited in a dominant fashion. All isogenic carp lines were subsequently used for infection studies. The genetic variation in susceptibility to the parasite *Trypanoplasma borreli* was dependent upon the immune response type. This suggested that the two parents (P20, P38) that were used to generate high- and low-responder homozygous families, differed for at least one gene with a major influence on resistance. Early/high-responder carp, as defined in the base population, showed a relative increase in representation of major histocompatibility complex (Mhc) class II β chain *Cyca-DAB1*01* and *Cyca-DAB2*01* linked genes, whereas low-responder carp showed a relative increase for *Cyca-DAB3*01* and *Cyca-DAB4*01* linked genes. The high- and low-responder F_1 hybrids differed with respect to these Mhc genes, which may have contributed to the observed differences in magnitude of immune responsiveness. Typically, these isogenic F_1 hybrid carp showed no within-line variation in DNA fingerprints.

Key words: *Cyprinus carpio* L., isogenic lines, DNA fingerprinting, disease model, immune response genes

Introduction

The genetic improvement of disease resistance might constitute an effective long-term measure to control infectious diseases in fish culture. Research has focussed on the search for those (non-) specific immune parameters that show genetic variation, and could have a genetic correlation with resistance to disease, two prerequisites for successful selection (Fjalestad *et al.*, 1993). For instance, genetic variation in complement haemolytic activity (Røed *et al.*, 1992, 1993), serum lysozyme levels (Røed *et al.*, 1993) as well as specific antibody production (Eide *et al.*, 1994; Strønsheim *et al.*, 1994a, b) has been described in the Atlantic salmon (*Salmo salar*). Genetic correlation of these parameters with disease resistance, however, has proven more difficult to establish. In general, the genetic variation in these immunological

traits explained too little variation in survival of diseases such as vibriosis and furunculosis, to be used in breeding schemes (Zarnecki *et al.*, 1994; Lund *et al.*, 1995a, b).

Fish have been shown to provide excellent laboratory models (Powers, 1989), although the available inbred lines are mostly from small-sized fish species such as the zebrafish (*Brachydanio rerio*; Westerfield, 1993) or medaka (*Oryzias latipes*; Hyodo-Taguchi and Egami, 1985). Their small size has limited their use for immunobiological studies. Ginbuna crucian carp (*Carrasius gibelio langsdorfii*) is a larger fish species for which clonal offspring is available, and has been used to investigate major histocompatibility complex (Mhc)-restricted cell transfer (Nakanishi and Onozato, 1990), but not for studies on the immunogenetic control of disease. So far, the availability of larger genetically-identical fish has remained limited, although the techniques to artificially produce these by gynogenesis have been described for a number of (commercially important) fish species (reviewed by Ihssen *et al.*, 1990).

We have chosen to produce isogenic lines of the common carp (*Cyprinus carpio* L.) via induced gynogenetic reproduction. The common carp, which is of high commercial value to many Eastern-European and Asian countries, and for which artificial gynogenesis has been described in detail (Nagy *et al.*, 1978; Komen *et al.*, 1989, 1991), is easy to reproduce under laboratory circumstances at which it can reach relatively large sizes within a few months. In combination with a well-described immune defense system (Rombout *et al.*, 1993; Stet *et al.*, 1993; Verburg-van Kemenade *et al.*, 1994), and defined disease models (Haenen and Davidse, 1993; Jones *et al.*, 1993; Daly *et al.*, 1994), reproduction of gynogenetic carp could result in isogenic fish lines for studies on the genetic control of cell-cell cooperation in the immune response to pathogenic organisms.

Antibody production to a hapten-carrier complex (dinitrophenyl-keyholelimpet haemocyanin; DNP-KLH) served as the immune parameter in order to divergently select carp with a genetically determined high or low immune responsiveness. Here, we describe the outcome of the selection, by examination of the antibody response to DNP-KLH in F_1 hybrids of homozygous carp. Furthermore, we examined the ubiquitous nature of the difference in immune responsiveness by immunization with other hapten-carrier complexes, and by an investigation of the response to infection with *Trypanoplasma borreli*. Previously, antibody production was shown to be important for protection in this particular parasite infection model (Wiegertjes *et al.*, 1995a). An attempt is made to explain the differences in immune responsiveness by examination of the isogenic nature of the F_1 hybrids using DNA fingerprint analysis, and by determination of Mhc genes present in the F_1 hybrids using previously described class II β-chain polymorphism.

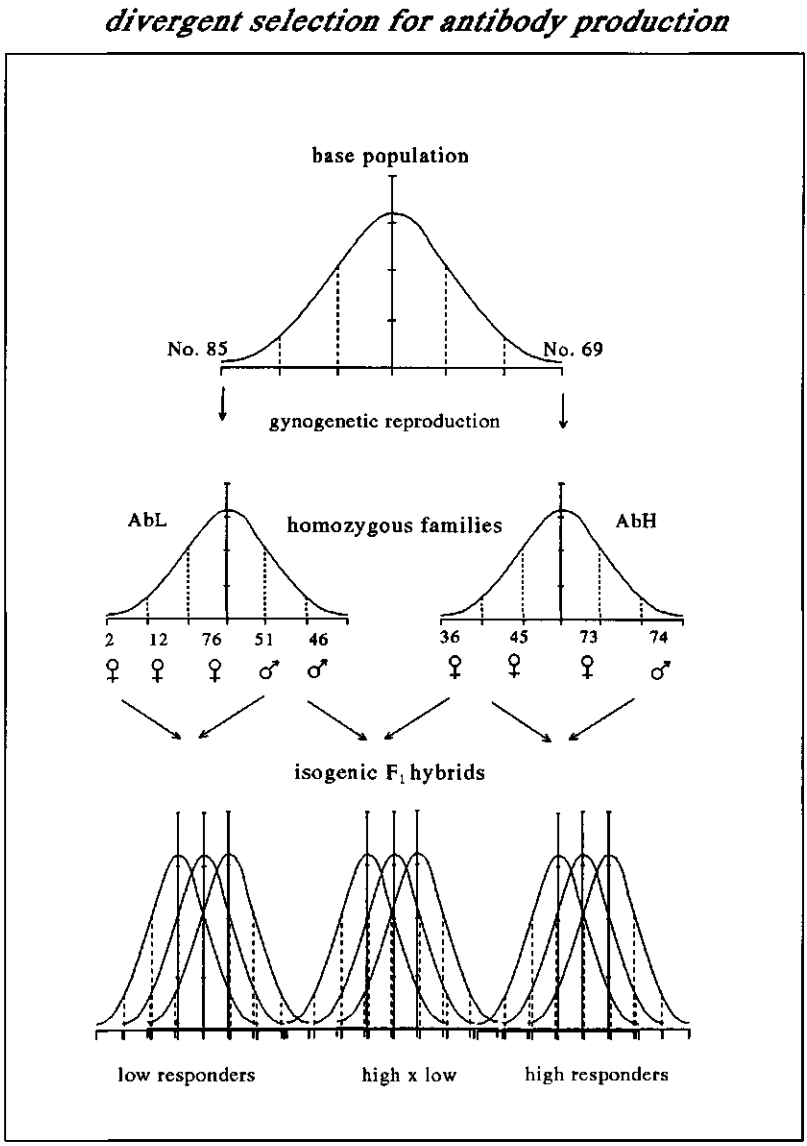


Figure 1. Divergent selection scheme applied to obtain high- and low-responder isogenic carp (*Cyprinus carpio* L.) lines. Two individuals from the base population; numbers 69 and 85, typed as high and low responder for antibody production to DNP-KLH, respectively, were gynogenetically reproduced to obtain homozygous families. Subsequently, homozygous males and females, denoted by their individual numbers, were crossed within, and between, the AbH and the AbL family, to obtain F_1 isogenic carp.

Materials and methods

Fish

A divergent selection for antibody production was initiated in a carp (*Cyprinus carpio* L.) base population, from which two individuals were taken and reproduced by gynogenesis, obtaining homozygous offspring (see figure 1). For information on the antibody response of carp from the base population, the reader is referred to Wiegertjes *et al.* (1994). The gonadal development of all carp from the two selected homozygous offspring, one with a high antibody response (from individual No. 69; further denoted as AbH family), and one with a low antibody response (from No. 85; AbL family), aged 16 months, was examined. The antibody response of these selected offspring has been described previously (Wiegertjes *et al.*, 1995c). Both families were shown to include reproductive homozygous females as well as functional males with the XX genotype (Bongers *et al.*, in preparation). The occurence of male sex reversal in gynogenetic female carp, which has been suggested to be induced by a recessive autosomal sex-determining gene (Komen *et al.*, 1992), allowed us to cross homozygous animals and obtain isogenic F_1 hybrids. Males and females from the AbH family were crossed to produce presumed high-responder carp, males and females from the AbL family to produce presumed low-responder carp. The females from the AbH family were also crossed with a male from the AbL family to produce high x low-responder carp (AbH x AbL, see figure 1).

The isogenic carp lines were grown at 25±2 °C in filtered and UV-treated water. After initial feeding on *Artemia* nauplii for a period of 3 weeks following yolk-sac absorption, the feeding regime was switched to a daily ration of 25 g $kg^{-0.8}$ (Provimi, Rotterdam, The Netherlands). All carp lines were produced within one week and were reared in separate aquaria of the same recirculation system. In general, fish were marked, immunized, or bled by caudal venepuncture, after anaesthesia in 0.02% (w/v) tricaine methane sulphonate (Crescent Research Chemicals, Phoenix, USA).

Immunization with hapten-carrier complexes

A number of animals (n=6-14 per antigen, see Table 1) were taken from each isogenic carp line. At 5 months of age, individuals were marked, mixed and randomly divided over two tanks. Carp were i.m. injected with either 10 μg T cell-dependent DNP-KLH, DNP- human serum albumin (HSA) or with T cell-independent trinitrophenyl-lipopolysaccharide (TNP-LPS), as described previously (Wiegertjes *et al.*, 1995b). Pre-immune serum samples were taken from 5-month-old fish, fish were immunized at 6 months. Blood samples were allowed to clot at room temperature for 1 h to obtain serum which was stored at -20 °C. DNP-specific serum antibody levels were measured 12, 21 and 28 days after immunization with an enzyme-

linked immunosorbent assay (ELISA; Wiegertjes *et al.*, 1994). In order to minimize plate differences, all samples were processed on a single day, after a slight modification of the ELISA procedure. In short, 96-well plates were coated overnight with DNP-bovine serum albumin (BSA) at 37 °C, and incubated overnight at 37 °C with 1% (w/v) BSA to block non-specific binding sites. Serum samples were serially two-fold diluted from 1:25 to 1:1600. Subsequent incubations with mouse monoclonal antibody against carp immunoglobulin (WCI-12) and goat-anti-mouse horseradish peroxidase were one hour at 37 °C. Ortho-phenylene-diamine (OPD) substrate incubation was stopped with sulphuric acid. Antibody titres were measured as optical density (OD) values at 492 nm at a fixed serum dilution (d=100), or as dilution factors corresponding to fixed OD values (OD=1.0) in regression equations.

Table 1. Immunization of 6-month-old F_1 isogenic carp (*Cyprinus carpio* L.) with T cell-dependent DNP-KLH or DNP-HSA, or with T cell-independent antigen TNP-LPS. Body weight was determined at the last sampling day (7 months).

		Experimental design			
family	cross	DNP-KLH	DNP-HSA	TNP-LPS	weight ± SD
	(♀ x ♂)	*n*	*n*	*n*	(g)
AbH (69)	36 x 74	10	10	10	234±74
	45 x 74	10	10	10	176±57
	73 x 74	10	10	10	101±34
AbH x AbL	36 x 46	10	10	10	246±96
	45 x 46	14	nd[1]	nd	150±61
	73 x 46	10	10	10	194±42
AbL (85)	2 x 51	8	8	8	112±63
	12 x 51	10	10	10	136±80
	76 x 51	12	6	7	114±62

[1] nd= not done

Parasite infection

Ten-month-old F_1 hybrid carp were infected with *Trypanoplasma borreli*, to study the resistance to the haemoflagellate parasite. To examine the influence of prior immunization with DNP-HSA at 6 months, non-immunized control fish were also infected (cross 73 x 46; AbH x AbL). Carp of each line were divided over two groups, of which one group was infected with virulent, and the other group with γ-irradiated parasites. Each group was placed in a separate 120 l aquarium of a flow-through system at 20±1 °C, and acclimated for four weeks. Averaged over all lines, carp weighed 230±158 g at infection.

Trypanoplasma borreli (Kinetoplastida: Cryptobiidae) was cloned and characterized by Steinhagen *et al.* (1989). Carp were i.m. infected with 1000 virulent, or with 5 x 10^4 γ-irradiated *T. borreli*. Irradiation-dose was 100 Gy, at a concentration of 5 x 10^5 ml^{-1}, to inactivate but not kill the parasite (G.F. Wiegertjes, unpublished data, 1994). A number of parameters were measured to establish possible differences in resistance to *T. borreli* between the carp lines. Parasitaemia was monitored by microscopical examination of heparinized blood samples at 3 weeks post-infection (wpi) with virulent parasites. Parasites were counted using a Bürker counting chamber. Dead fish were recorded and removed daily for 6 weeks. Blood samples to measure serum antibody titres, elicited by injection with irradiated *T. borreli*, were taken 5 wpi. Specific antibody production was measured with an ELISA (Jones *et al.*, 1993).

DNA-fingerprinting

High molecular weight carp DNA was obtained from liver, or from nuclei of lysed erythrocytes, from (n=5) individuals of all hybrid crosses. DNA isolation and digestion were as described previously (Stet *et al.*, 1993). Briefly, homogenized samples were incubated overnight in TEN (100 mM Tris/HCl pH 8.0, 10 mM EDTA, 250 mM NaCl) buffer containing 1% (w/v) SDS, at 37°C, in the presence of proteinase-K (1 mg/ml). DNA was extracted by phenol, chloroform/iso-amyl alcohol (IAA), and precipitated in 0.8 vol. 2-propanol at -20°C. This precipitate was dissolved in TE (10 mM Tris/HCl pH 7.6, 1 mM EDTA) buffer, and finally precipitated in sodium acetate and 100% ethanol (-20°C). The DNA was washed in 70% ethanol, dried, and dissolved in TE buffer. DNA samples (50 µg) were digested in a large volume (500 µl) with *Hae*III or with *Hinf* I (4 units μg^{-1} DNA), according to the manufacturer's (GIBCO, BRL) specifications, in the presence of RNAse. Digested DNA samples (10 µg/lane) were separated on 0.8% agarose gels, run at 4°C for 960 Vh in recirculating buffer. After electrophoresis, gels were transferred to nylon filters (Hybond-N+, Amersham, UK) using a vacuum-blot system (Pharmacia, Uppsala, Sweden). Fingerprinting was essentially performed as described by Johnstone and Stet (1995). The microsatellite oligonucleotide probe $(GGAT)_4$ was end-labelled using polynucleotide kinase according to

specification. Filters were prehybridized overnight at 45°C in 5 x SSC, 1% SDS, 5 mM EDTA, 5 x Denhardt's and 20 μg ml^{-1} denatured *E. coli* DNA. Hybridization was performed overnight at 45°C, in the same mixture, by adding $1x10^6$ cpm/ml labelled probe. Filters were washed with 5 x SSC, 0.2% SDS at room temperature followed by 10 min. at 46°C. Autoradiography was performed at -70°C using Fuji-RX or Amersham Hyperfilm-MP imaging films with intensifying screens, or at -20°C using Amersham Hyperfilm β-max without screens.

Mhc class II genotyping for *Cyca-DAB*

Polymerase chain reaction (PCR) amplification was performed on 100-200 ng genomic DNA from the two parents of the base population (female No. 38, R8 strain; male No. 20, R3 strain), by combining primers based on *Cyca-DAB* cDNA sequences codon -4 to 4 (OL93-139 5'-CTGTCTGCTTTCACTGGAGCAG-3') and codon 89 to 96 (OL93-140 5'-CTGTTTTATC ACGGATCGCCGA-3'), or codon 80 to 88 (OL93-23 5'-CTGATAGAGTTCAGCATTATGT TTGCA-3') (Ono *et al.*, 1993), with 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Eurogentec, Seraing, Belgium) and reaction buffer in a final volume of 100 μl. The mixture was subjected to a thermal cycle profile (1 min 94 °C, 2 min 55 °C, 1 min 72 °C) for 30 cycles. The PCR products were cloned into PGEM-TA (Promega, Madison, WI) and sequenced using the T7 DNA polymerase version 2.0 (USB, Cleveland, USA). Segregation studies using the above described techniques have indicated that *Cyca-DAB*01* and *Cyca-DAB*02* are in fact closely linked genes, further designated as *Cyca-DAB1*01* and *Cyca-DAB2*01*, respectively (van Erp *et al.*, in preparation).

Genomic DNA from n=40 fish from the base population, including the two individuals Nos 69 and 85 that were used to generate the AbH and AbL families, and genomic DNA from the homozygous individuals used for the F_1 hybrids, was used for *Cyca-DAB* genotyping. Genes were detected using two techniques. 1. PCR-RFLP and 2. gene-specific PCR. PCR-RFLP uses restriction enzyme digestion of the resultant PCR product, generating differently sized fragments. For this purpose primers OL93-139 and OL93-140 were used to generate a *Cyca-DAB1*01/Cyca-DAB2*01* fragment in a standard PCR as described above. The PCR mix (20 μl) was digested with *Rsa*I, and separated on 1% agarose gel. Gene-specific PCR uses primers specific for the PCR amplification of known *Cyca-DAB* sequences of predictable size. In this study *Cyca-DAB3*01* (codons 78-84; 5'-CTCTGCTGCAGTTCTGCC-3') and *Cyca-DAB4*01* (codons 84-90; 5'-TGTCCACTGAAGTTTTCAGA-3') were used to amplify fragments of 746 bp and 678 bp, respectively. These genes were also shown to be linked (van Erp *et al.* , in preparation). PCR amplifications were done twice on different occasions to minimize detection of false positives or negatives, and PCR samples were analyzed by agarose gel electrophoresis.

Results

Hapten-carrier immunization

The kinetics of the antibody response to the various hapten-carrier complexes were seen to be different. For comparison, the antibody production of one high-responder, and one low-responder line, representative for the other F_1 hybrids, is shown (Fig. 2). The increase in antibody production to T cell-dependent DNP-HSA (Fig. 2b) was slower than seen after immunization with DNP-KLH (Fig. 2a). In contrast, the response to T cell-independent TNP-LPS (Fig. 2c) was more rapid than to either T cell-dependent antigen (*cf.* 12 days), although the response increased slightly after 12 days (*cf.* 28 days). It was decided, based upon the differences in kinetics and magnitude of response, to compare the immune responsiveness of the isogenic lines at 21 days after immunization with DNP-KLH, at 28 days after immunization with DNP-HSA and at 12 days after immunization with TNP-LPS.

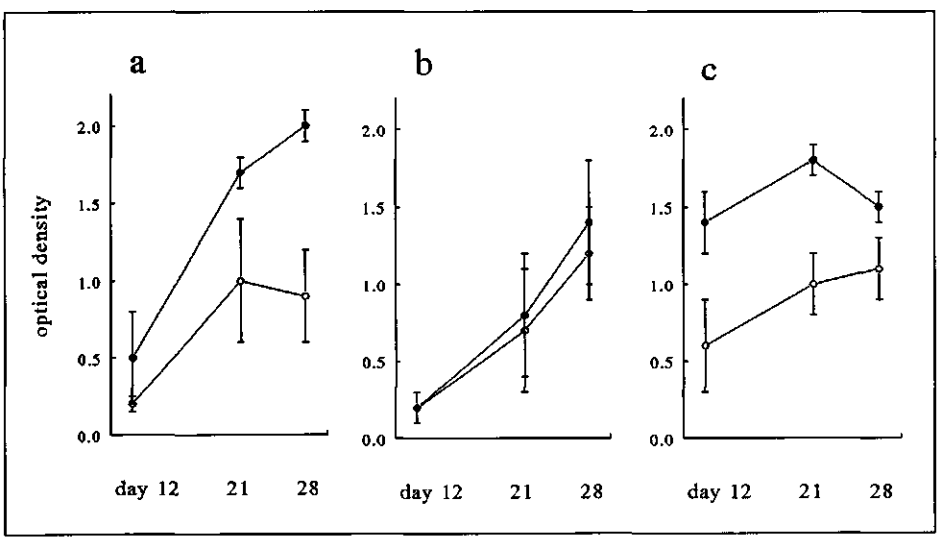


Figure 2. Kinetics of the DNP-specific antibody response of F_1 hybrid carp. The antibody production of one high-responder (●: 73 x 74; AbH family) and one low-responder line (○: 76 x 51; AbL family), is visualized. Fish were immunized with (**a**) DNP-KLH, (**b**) DNP-HSA, or (**c**) with TNP-LPS. Pre-immune values (0.1-0.2 OD) are not shown.

Table 2. Antibody response to DNP-KLH of presumed high- and low-responder isogenic F_1 hybrid carp. Antibody titres are expressed as optical density (OD) values of 1:100 diluted serum samples.

Parental immune response types		Antibody response isogenic lines (mean OD ± SD)			
Family	Cross (♀ x ♂)	pre-immune	12 days	21 days	28 days
AbH (69)	36 x 74	0.1±0.1	0.3±0.1	1.5±0.2	1.7±0.2
	45 x 74	0.2±0.1	0.3±0.1	1.2±0.4	1.5±0.2
	73 x 74	0.1±0.1	0.5±0.3	1.7±0.1	2.0±0.1
AbL (85)	2 x 51	0.1±0.1	0.2±0.0	1.2±0.3	1.4±0.3
	12 x 51	0.1±0.1	0.2±0.1	1.1±0.3	1.5±0.3
	76 x 51	0.1±0.1	0.2±0.0	1.0±0.4	0.9±0.3

It was found that the antibody production to DNP-KLH of presumed low-responder carp, was lower than the antibody response of presumed high-responder carp (Table 2). This was seen at all sample days after immunization. The antibody titre differences of maximally 0.7 OD (at 21 days; see Table 2), when converted into serum dilution factors in regression equations, corresponded to approximate 10-fold differences in serum dilutions. Immune sera from high-responder lines could be 1000-fold diluted, compared with immune sera from the low-responder line, which could be approximately 100-fold diluted, to obtain identical OD-values in the ELISA (Fig. 3a). Plotting the frequency distributions of the antibody response to DNP-KLH (Fig. 3b), a difference in immune responsiveness between the presumptive high- and low-responder lines could be seen. In contrast, the frequency distribution of the AbH x AbL lines was similar to that of the high-responder carp (see Fig. 3b).

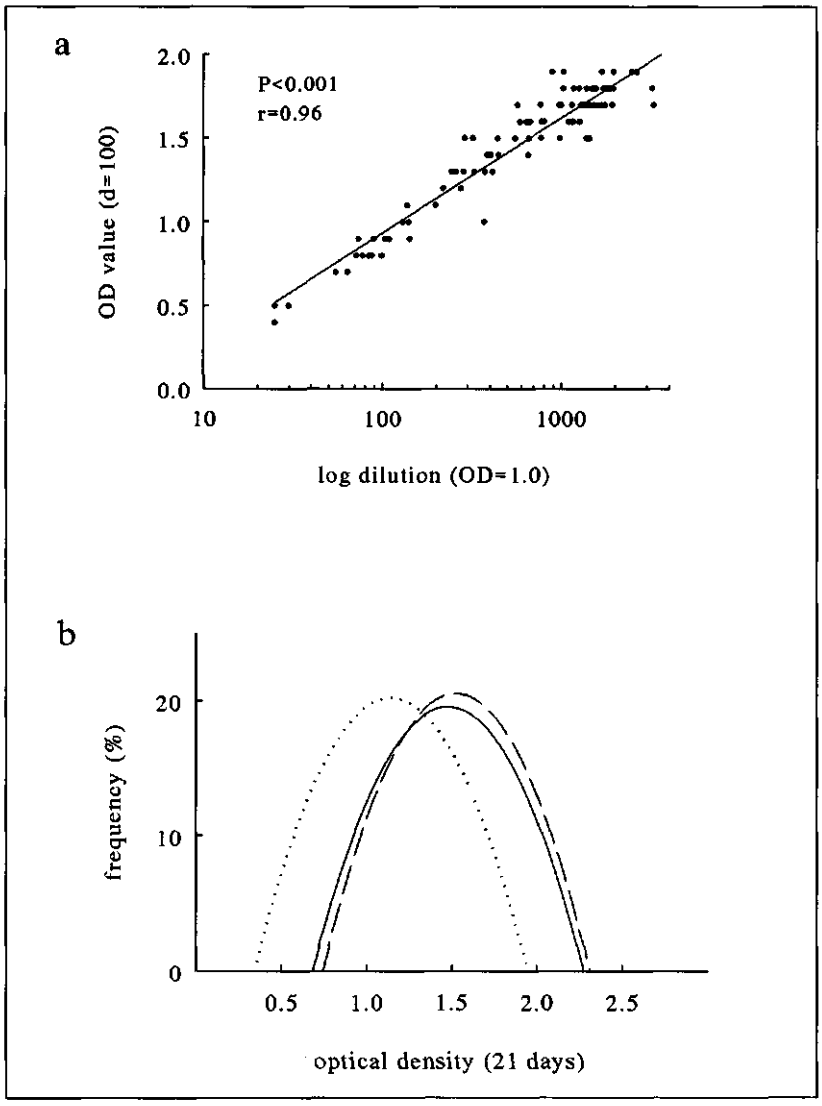


Figure 3a. Correlation between DNP-specific antibody titres, calculated as optical density (OD) values at fixed serum dilutions (d=100), or as serum dilution factors at a fixed optical density (OD=1.0), at 21 days after immunization with DNP-KLH. **b.** Frequency distributions of the DNP-specific antibody response to DNP-KLH (21 days) of presumed high-responder (continuous line; AbH family), presumed low-responder carp (dotted line; AbL family), and AbH x AbL carp (dashed line). Values were averaged over three isogenic F_1 hybrid crosses per group.

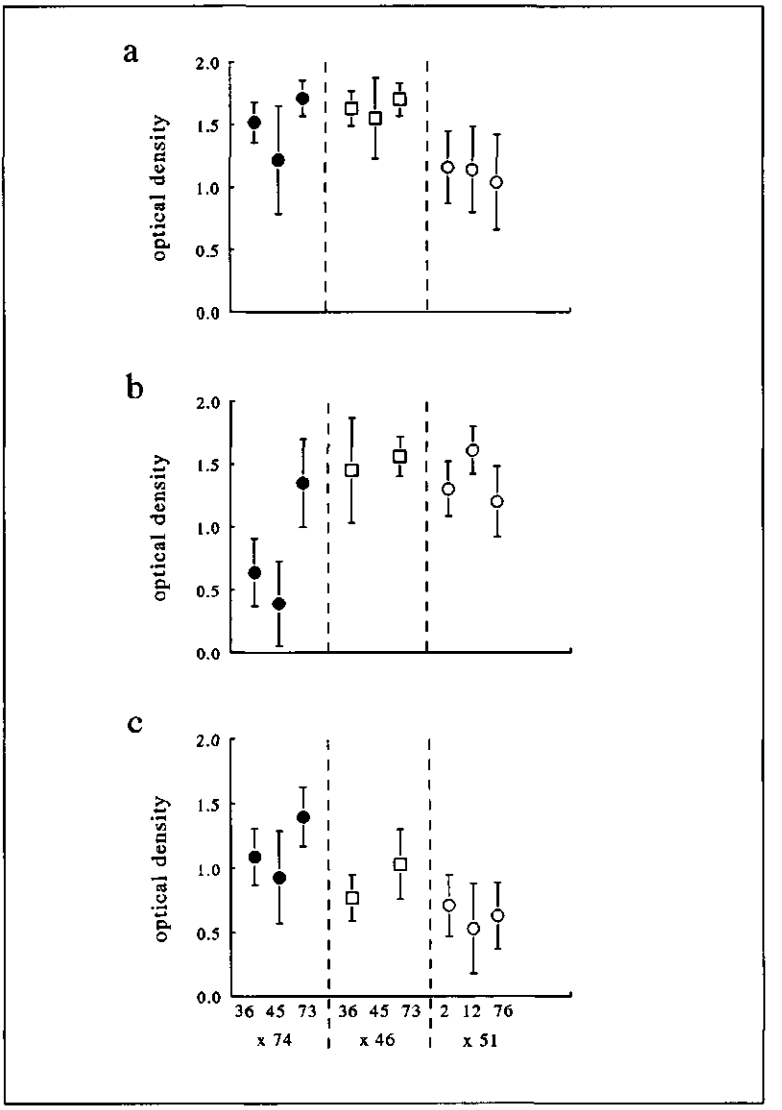


Figure 4. a-c: Antibody titres (mean optical density ± SD) of presumed high-responder (●: AbH family), presumed low-responder (○: AbL family) and high x low-responder (□: AbH x AbL) isogenic carp. According to observed differences in kinetics and magnitude of the response, antibody production is visualized (**a**) at 21 days for DNP-KLH, (**b**) at 28 days for DNP-HSA, and (**c**) at 12 days for immunization with TNP-LPS.

Infection with *Trypanoplasma borreli*

Three weeks after infection, the variation in (average) parasitaemia between all isogenic carp lines tested was 37-fold (1×10^6 - 3.7×10^7), with the highest parasitaemia detected in carp from the AbL family. The lowest parasitaemia was detected in carp from both the AbH family and in AbH x AbL carp. Parasitaemia was negatively correlated with survival time and total survival of the carp. Furthermore, in general, isogenic carp lines from the AbH family showed increased antibody production specific to *T. borreli*, whereas carp from the AbL family often failed to react to the parasite (Table 3).

There was a slight difference in survival between previously immunized and non-immunized control fish, but no differences in any of the other parameters measured (data not shown). No correlation could be detected between the magnitude of response to DNP-HSA and subsequent resistance to *T. borreli*.

Table 3. Resistance to infection with *Trypanoplasma borreli* of isogenic F_1 hybrid carp. Resistance was measured as parasitaemia (*T. borreli* ml^{-1} blood) 3 weeks post-infection (wpi) and as survival time, both presented as individual values, or as survival (number of survivors/infected fish) at 6 wpi. The last column shows the relative number of animals with increased specific antibody titres (≥ 1:320), measured at 5 wpi with γ-irradiated trypanoplasms.

Experimental design		Resistance to *T. borreli*			
Family	Cross (♀ x ♂)	Parasitaemia ($\times 10^6$)	Survival time (days)	Survival	Increased Ab titres
AbH	36 x 74	1 1 1 1 2	>42 >42 >42 >42 >42	5/5	4/4
	45 x 74	1 1 2 2 4	30 32 35 39 >42	1/5	1/5
	73 x 74	1 1 3 4 7	39 >42 >42 >42 >42	4/5	4/5
AbH x AbL	36 x 46	1 1 1 1	33 36 37 >42	1/4	5/5
	45 x 46	nd[1]	-	-	-
	73 x 46	1 2 2 4	33 33 >42 >42	2/4	3/4
AbL	2 x 51	8 22	28 32	0/2	0/3
	12 x 51	2 2 4 7	28 31 31 32	0/4	0/3
	76 x 51	1 29 81	27 31 31	0/3	1/3

[1] nd= not done

Genetic effects

The total variation (phenotypic variance V_P) of the full sibs in the base population, comprised the additive effects of genetic (V_G) and environmental (V_E) variance. In general, V_G can be divided into additive (V_A) and non-additive genetic variation, the latter including dominance effects (V_D). Each homozygous family consisted of animals generated from one single mother, all with a coefficient of coancestry $f_{x,y}=1$ and thus an additive genetic relation of $a_{x,y}=2f_{x,y}=2$ (see also Table 4). These families have no dominance variance, because of homozygosity, but may suffer from developmental instability leading to an increase in V_E. The isogenic carp lines, by definition, consist of genetically-identical animals (genetic variance $V_G = 0$), with dominance effects equal for all individuals within one line, and no increased environmental variation due to heterozygosity (no developmental instability).

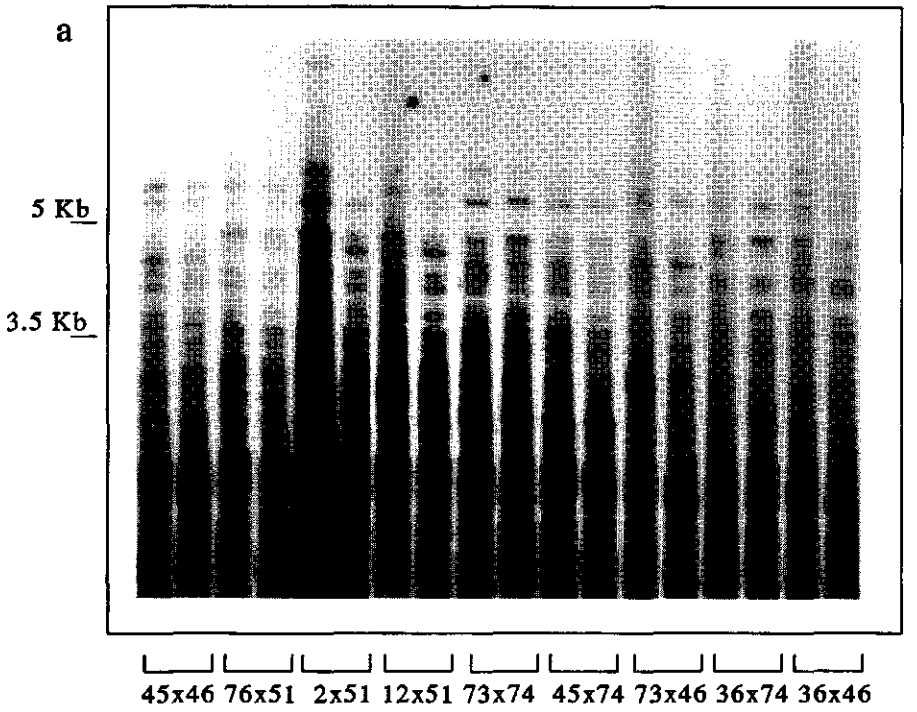
Owing to the positive selection response ($h^2 = 0.29$-0.37; Wiegertjes *et al.*, 1994, 1995c), a reduction of the phenotypic variance for antibody production to DNP-KLH of approximately 30 per cent could be expected within the isogenic carp lines. Examination of the observed variance indicated no reduction within the homozygous families, but a clear reduction of V_P within the isogenic F_1 hybrids, compared with base population carp (Table 4). For the analysis of animal breeding experiments, an animal model is the method of choice (Meyer, 1989). This analysis, however, does not allow for the homozygous, but all different, nature of the individuals that comprise a homozygous family, and assumes that variances are equal. Differences in assay conditions, in age, and in kinetics of the antibody response, between the selection steps, however, affect the phenotypic variances differently.

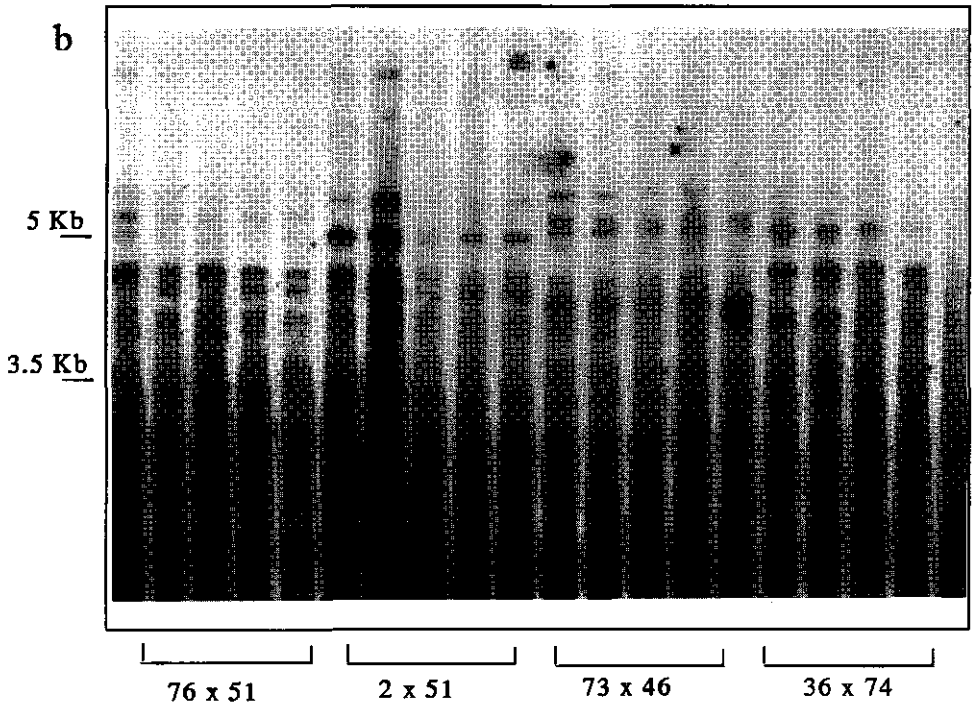
Table 4. Expected variance components and observed phenotypic variance (V_P) throughout the divergent selection for antibody production (OD values), at 12, 21 and 28 days after immunization with DNP-KLH.

Group	Age (months)	Components V_P	Observed variance		
			12 days	21 days	28 days
Base population	13	$V_A + V_D + V_E$	0.19	0.20	nd[1]
Homozygous families[2]	6	$2V_A + V_E$	0.02	0.30	0.24
Homozygous families[3]	12	$2V_A + V_E$	0.08	0.26	0.19
F_1 hybrids[4]	6	V_E	0.02	0.08	0.06

[1] nd = not done
[2] variance averaged over two families (high + low); ref. Wiegertjes *et al.*, 1994
[3] variance averaged over two families (high + low); ref. Wiegertjes *et al.*, 1995c
[4] F_1 *hybrids: variance averaged over nine isogenic carp lines*

To scrutenize the absence of genetic variation within the F_1 isogenic lines, genomic DNA from all hybrid crosses was subjected to fingerprint analysis. The oligonucleotide probe $(GGAT)_4$ yielded multibanded fingerprints with a reasonable number of scorable fragments. The most useful enzyme for fingerprint determination was *Hinf* I, since digestion with *Hae*III could not detect between-line genetic heterogeneity (data not shown). The number of scorable bands in a fingerprint phenotype varied with the isogenic line examined, but on average, *Hinf* I digestion yielded 10-14 scorable fragments. Fingerprints were seen to be different between the F_1 hybrids (Fig. 5a), but identical within the isogenic lines (Fig. 5b).





Left page: **Figure 5a.** DNA fingerprints of two individuals from each isogenic F_1 hybrid cross, obtained with the enzyme *Hinf* I and $(GGAT)_4$ microsatellite probe. The different crosses are denoted by the numbers of the two homozygous parents, as described in Table 1. *Above:* **b.** DNA fingerprints of five individuals from four representative isogenic F_1 hybrids. Sizes of molecular weight standards are given in kilobases; their positions have been derived from the locations of bands on ethidium bromide-stained gels photographed prior to probe hybridization.

Mhc class II genotyping

Genomic DNA from the two parents of the base population was amplified with PCR using primers based on *Cyca-DAB* cDNA sequences. The PCR-products were cloned and sequenced. *Cyca-DAB1*01/DAB2*01* linked genes were detected in both parents (P20, P38), whereas *Cyca-DAB3*01/DAB4*01* linked genes were present in the female parent only (P38, R8 strain). PCR-RFLP and gene-specific PCR analyses of genomic DNA from carp of the base population demonstrated the presence of three different *Cyca-DAB* genotypes (see Table 5).

Both combinations of linked-genes segregated in the base population, in a 3:2 ratio of *Cyca-DAB1*01/DAB2*01* to *Cyca-DAB3*01/DAB4*01* (expected frequencies).

In association with the immune response types previously described for base population carp (Wiegertjes *et al.*, 1994), a relative decrease in the frequence of *Cyca-DAB1*03/DAB2*04* genes was observed within the early/high responder group, and a relative increase in the medium responder group of *Cyca-DAB1*01/DAB2*01* genes (observed frequencies), whereas expected and observed frequencies were similar in the low responder group.

The female parent of the AbH family (No. 69) was typed as *Cyca-DAB1*01/DAB2*01*, and all individuals from this family tested, were homozygous for these genes, which indicated an apparent homozygosity of carp No. 69 for *Cyca-DAB1*01/DAB2*01*. The female parent of the AbL family (No. 85) was typed as *Cyca-DAB3*01/DAB4*01*. Some (Nos 12, 46, 51), but not all (76, 2 not tested) individuals from this family tested, were homozygous for these genes, indicating the presence of a null allele in carp No. 85. All presumed high-responder carp were homozygous for *Cyca-DAB1*01/DAB2*01*, whereas all AbH x AbL hybrids carried both *Cyca-DAB1*01/DAB2*01* and *Cyca-DAB3*01/DAB4*01* genes. All presumed low-responder hybrids at least had the *Cyca-DAB3*01/DAB4*01* genes.

Table 5. Expected and observed numbers of individuals with different *Cyca-DAB* genotypes in association with immune response types defined in the base population (n = 40).

Mhc genotype	Early/high resp.		Late/low resp.		Medium resp.	
Cyca-DAB	Exp	Obs	Exp	Obs	Exp	Obs
*1*01/2*01*	4.5	4	4	4	11.5	16
*1*01/2*01 3*01/4*01*	2.25	5	2	2	5.75	2
*3*01/4*01*	2.25	0	2	2	5.75	5

Discussion

The F_1 hybrids of homozygous carp were the result of a long-term divergent selection for antibody production to DNP-KLH (Wiegertjes *et al.*, 1994, 1995c). Previously, we have examined the ubiquitous nature of the immune response types in selected homozygous families, by studying the antibody response to hapten-carrier complexes other than DNP-KLH (Wiegertjes *et al.*, 1995b). In addition to these defined antigens, the resistance to a haemoflagellate parasite *Trypanoplasma borreli* (see Wiegertjes *et al.*, 1995a), was investigated. In the present chapter, we have examined the immune responsiveness of the selected high- and low-responder F_1 isogenic carp using the same experimental design.

In order to generate isogenic lines with presumed high responsiveness to DNP-KLH, homozygous individuals from the AbH family were crossed. Similarly, individuals from the AbL family were crossed to produce carp lines with presumed low responsiveness. The limited availability of functional males reduced the possibility to select within each family, however. The antibody titres to DNP-KLH were higher in the high-responder, than in the low-responder carp. Immune sera from the high-responders could be 1000-fold diluted, compared with immune sera from the low-responders, which could be 100-fold diluted only, to obtain identical optical densities in a DNP-specific ELISA. Furthermore, the magnitude of the antibody production in crosses between high and low-responder carp suggested that high responsiveness was inherited in a dominant fashion. In accordance with the previously suggested genetic control of the antibody response to DNP-KLH (h^2 = 0.29-0.37; Wiegertjes *et al.*, 1994, 1995c), the phenotypic variation within the F_1 hybrids ($V_P = V_E$) in antibody production to DNP-KLH, was reduced in comparison with the base population and homozygous families. Little is known about the variation in immune responsiveness of isogenic fish lines, but a reduction of phenotypic variation in length and body weight in F_1 hybrids between homozygous carp has been reported (Komen *et al.*, 1993). The residual within-line variation could not be explained by technical errors during the gynogenetic reproduction, and can be considered unbiased estimates of the environmental variation. The females (Nos 69 and 85), that were reproduced gynogenetically to obtain the AbH and AbL families, were homozygous for a recessive gene inhibiting normal scalation. The males donating milt for UV-irradiation, needed to activate the eggs in gynogenetic reproduction, were animals homozygous scaled, which is the dominant trait. The subsequent absence of scaled fry provided a check for the absence of paternal genes in the assumptive homozygous families (Komen, 1990). More definite proof was the absence of within-line variation in DNA fingerprints, a technique which has also been used to establish clonal stability of natural gynogenetic *Poecilia formosa* (Turner *et al.*, 1990).

It was shown that antibody production to T cell-dependent DNP-HSA did not always follow the same pattern of response to DNP-KLH. Immunization with T-independent TNP-LPS had no effect on the response type. These observations are consistent with previous findings in high- and low-responder families (Wiegertjes *et al.*, 1995b), and suggest a carrier, or T cell-dependency of the divergent differences in responsiveness. Immunization with a nitrophenyl-unrelated hapten coupled to the KLH carrier, could possibly confirm this hypothesis.

Despite their common environment, the growth performances differed between the isogenic lines examined (see Table 1), apparently dependend upon the specific combining ability of the two genotypes used to produce each hybrid cross, more than on the AbH or AbL nature of their background. Although all carp were immunized with the same antigen dose, low-responder carp lines had often, but not always, lower body weights than high-responder carp. Previously, in carp from the base population, a positive relation between body weight and antibody titre at 12 days, suggested that weight differences could result in a slower response in larger animals (Wiegertjes *et al.*, 1994). Consequently, if body weight has influenced the antibody response in the isogenic lines, even larger differences could be expected between high- and low-responder carp with identical body weights.

The haemoflagellate infection model, for which a genetic control of susceptibility to the disease has been suggested to be caused by an impaired humoral response to the parasite (Wiegertjes *et al.*, 1995a), was used to further investigate the ubiquitous nature of the immune responsiveness of the selected carp lines. A number of parameters were measured, in order to find clear-cut differences in disease resistance. In general, F_1 hybrid lines from the AbL family were more susceptible than carp from the AbH family, whereas the AbH x AbL hybrids were high responders, indicating dominance. The differences could not only be visualized by differences in parasitaemia and survival time or total survival, but also in the ability to produce specific antibodies to the parasite. Interestingly, a similar failure to respond with a significant increase in antibody production to both DNP-KLH and *T. borreli* was previously found in carp lines unrelated to the selected lines described here (Wiegertjes *et al.*, 1995a). The present results clearly suggest that the two parents (Nos 69 and 85) selected to generate the AbH and AbL families, differed for at least one gene with a major influence on resistance to *T. borreli.* Particular Mhc haplotypes of the mouse have been implicated to play a major role in increased susceptibility to a number of parasitic diseases. For instance, the Mhc class II $I\text{-}E^k$ haplotype in mice has been linked to increased susceptibility to *Trichinella spiralis*, *Leishmania donovani* (Wassom and Kelly, 1990), and *Trypanosoma cruzi* (Powell and Wassom, 1993).

The antigen-dependent switch from high responsiveness (to DNP-KLH) to low responsiveness (to DNP-HSA) in some of the isogenic lines, and *vice versa*, is typical for immune response gene control, as first described for the mouse by McDevitt and Sela (1965). Although, at this point, it is premature to link the observed differences in resistance to *T. borreli* to Ir gene control, the initial choice for DNP-KLH as selection antigen was based upon the often observed correlation between antibody production to hapten-carrier complexes and immune response (Ir) genes of the Mhc (Klein, 1986). The suggested dependency of the differences in immune responsiveness on the carrier, which antigenic determinants are presented to the T cell by Mhc encoded molecules, suggests that Ir genes could play a major role in determining the differences in response to DNP-KLH between the selected carp lines. This prompted an investigation into the segregation of carp Mhc class II genes throughout the divergent selection procedure.

Four previously described *Cyca-DAB* genes could be detected in the original parents (P38, P20), resulting in three different genotypes within the base population. Early/high responsiveness was associated with a relative increase in frequency of the linked *Cyca-DAB1*01/DAB2*01* genes, whereas late/low responsiveness was associated with a relative increase in frequency of the linked *Cyca-DAB3*01/DAB4*01* genes. The latter genes are characterized by an aberrant splice site sequence for intron 1, in which GT is replaced by GC, which is highly infrequent throughout evolution (0.6% of the total gene sequences studied; Jacob and Gallinaro, 1989). A similar substitution has been shown to cause incomplete splicing of a high percentage of the primary transcripts of complement component C5 (Haviland *et al.*, 1991). This could result in lower expression levels of these Mhc molecules, encoded by genes carrying the aberrant splice site, on the cell surface, which may be the reason for the low immune response types observed. Despite the large increase in information on teleost Mhc class II genes (Dixon *et al.*, 1995), this is the first study that reports on an association of Mhc class II loci with the magnitude of immune responsiveness in fish.

Acknowledgements

T. borreli was kindly provided by Dr. D. Steinhagen, Hannover, and the R3 and R8 carp by the Fish Culture Experimental Station Golysz. This research was financially supported by the Netherlands Technology Foundation (SLW), and was coordinated by the Life Sciences Foundation (SLW). The authors wish to thank Drs. J.A.M. van Arendonk and H. Bovenhuis from the Department of Animal Breeding for helpful discussions on the genetic effects.

References

Daly, J.G., Wiegertjes, G.F. and van Muiswinkel, W.B. 1994. Protection against carp erythrodermatitis following bath or subcutaneous exposure to sublethal numbers of virulent *Aeromonas salmonicida* subsp. *nova*. J. Fish Dis. 17, 67-75.

Dixon, B., van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. 1995. Fish major histocompatibility complex genes: an expansion. Dev. Comp. Immunol. 19, 109-133.

Eide, D.M., Linder, R.D., Strønsheim, A., Fjalestad, K.T., Larsen, H.J.S. and Røed, K.H. 1994. Genetic variation in antibody response to diphtheria toxoid in Atlantic salmon and rainbow trout. Aquaculture 127, 103-113.

Fjalestad, K.T., Gjedrem, T. and Gjerde, B. 1993. Genetic improvement of disease resistance in fish : an overview. Aquaculture 111, 65-74.

Haenen, O.L.M. and Davidse, A. 1993. Comparative pathogenicity of two strains of pike fry rhabdo virus and spring viremia of carp virus for young roach, common carp, grass carp and rainbow trout. Dis. Aquat. Org. 15, 87-92.

Haviland, D.L., Haviland, J.C., Fleisher, D.T. and Wetsel, R.A. 1991. Structure of the murine fifth complement component (C5) gene. A large, highly interrupted gene with a variant donor splice site and organizational homology with the third and fourth complement component genes. J. Biol. Chem. 18, 11818-11825.

Hyodo-Taguchi, Y. and Egami, N. 1985. Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. Zool. Sci. 2, 305-316.

Ihssen, P.E., McKay, L.R., McMillan, I. and Phillips, R.B. 1990. Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Trans. Am. Fish. Soc. 119, 698-717.

Jacob, M. and Gallinaro, H. 1989. The 5' splice site: phylogenetic evolution and variable geometry of association with U1RNA. Nucl. Acids Res. 17, 459-472.

Johnstone, R. and Stet, R.J.M. 1995. The production of gynogenetic Atlantic salmon, *Salmo salar* L. T.A.G. 90, 819-826.

Jones, S.R.M., Palmen, M. and van Muiswinkel, W.B. 1993. Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. Vet. Immunol. Immunopathol. 36, 369-378.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley & Sons, New York, NY, USA.

Komen, J. 1990. Clones of common carp. New perspectives in fish research. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Komen, J., Bongers, A.B.J., Richter, C.J.J., van Muiswinkel, W.B. and Huisman, E.A. 1991. Gynogenesis in common carp (*Cyprinus carpio* L.). II. The production of homozygous gynogenetic clones and F_1 hybrids. Aquaculture 92, 127-142.

Komen, J., de Boer, P. and Richter, C.J.J. 1992. Male sex-reversal in gynogenetic XX females of common carp (*Cyprinus carpio* L.) by a recessive mutation in a sex-determining gene. J. Hered. 83, 431-434.

Komen, J., Duynhouwer, J., Richter, C.J.J. and Huisman, E.A. 1989. Gynogenesis in common carp (*Cyprinus carpio* L.). I. Effects of genetic manipulation of sexual products and incubation conditions of eggs. Aquaculture 69, 227-239.

Komen, J., Eding, E.H., Bongers, A.B.J. and Richter, C.J.J. 1993. Gynogenesis in common carp (*Cyprinus carpio* L.). IV. Growth, phenotypic variation and gonad differentiation in normal and methyltestosterone-treated homozygous clones and F_1 hybrids. Aquaculture 111, 271-280.

Lund, T., Chiayvareesajja, J., Larsen, H.J.S. and Røed, K.H. 1995a. Antibody response after immunization as a potential indirect marker for improved resistance against furunculosis. Fish Shellf. Immunol., in press.

Lund, T., Gjedrem, T., Bentsen, H.B., Eide, D.M., Larsen, H.J.S. and Røed, K.H. 1995b. Genetic variation in immune parameters and associations to survival in Atlantic salmon. J. Fish Biol. 46, 748-758.

McDevitt, H.O. and Sela, M. 1965. Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. J. Exp. Med. 122, 517-531.

Meyer, K. 1989. Estimation of genetic parameters. In: Evolution and animal breeding (eds: Hill, W.H. and Mackay, T.F.C). C.A.B. Int., Wallingford, UK, pp. 161-167.

Nakanishi, T. and Onozato, H. 1990. Clonal ginbuna crucian carp as a model for the study of fish immunology and genetics. Genetics in Aquaculture. In: Proceedings of the 16th US-Japan meeting on aquaculture (ed: Svrjcek, R.S). NOAA Techn. Report NMFS 92, Washington, USA.

Nagy, A., Rajki, K., Horvath, L. and Csanyi, V. 1978. Investigation on carp, *Cyprinus carpio* L., gynogenesis. J. Fish Biol. 13, 215-224.

Ono, H., O'hUigin, C., Vincek, V., Stet, R.J.M., Figueroa, F. and Klein, J. 1993. New β chain-encoding *Mhc* class II genes in the carp. Immunogenetics 38, 146-149.

Powell, M.R. and Wassom, D.L. 1993. Host genetics and resistance to acute *Trypanosoma cruzi* infection in mice. I. Antibody isotype profiles. Parasite Immunol. 15, 215-221.

Powers, D.A. 1989. Fish as model systems. Science 246, 352-358.

Røed, K.H., Fjalestad, K.T., Larsen, H.J. and Midthjel, L. 1992. Genetic variation in haemolytic activity in Atlantic salmon (*Salmo salar*). J. Fish Biol. 40, 739-750.

Røed, K.H., Fjalestad, K.T. and Strønsheim, A. 1993. Genetic variation in lysozyme activity and spontaneous haemolytic activity in Atlantic salmon (*Salmo salar*). Aquaculture 114, 19-31.

Rombout, J.H.W.M., Taverne, N., van de Kamp, M. and Taverne-Thiele, A.J. 1993. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 17, 309-317.

Steinhagen, D., Kruse, P. and Körting, W. 1989. The parasitaemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). J. Parasitol. 75, 685-689.

Stet, R.J.M., van Erp, S.H.M., Hermsen, T., Sültmann, H.A. and Egberts, E. 1993. Polymorphism and estimation of the number of *MhcCyca* class I and class II genes in laboratory strains of the common carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. 17, 141-156.

Strønsheim, A., Eide, D.M., Fjalestad, K.T., Larsen, H.J.S. and Røed, K.H. 1994a. Genetic variation in the humoral immune response in Atlantic salmon (*Salmo salar*) against *Aeromonas salmonicida* A-layer. Vet. Immunol. Immunopathol. 41, 341-352.

Strønsheim, A., Eide, D.M., Hofgaard, P.O., Larsen, H.J.S., Refstie, T. and Røed, K.H. 1994b. Genetic variation in the humoral immune response against *Vibrio salmonicida* and in antibody titre against *Vibrio anguillarum* and total IgM in Atlantic salmon (*Salmo salar*). Vet. Immunol. Immunopathol. 44, 85-95.

Turner, B.J., Elder Jr., J.F., Laughlin, F. and Davis, W.P. 1990. Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting. Proc. Natl. Acad. Sci. USA 87, 5653-5657.

Verburg-van Kemenade, B.M.L., Groeneveld, A., van Rens, B.T.T.M. and Rombout, J.H.W.M. 1994. Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). J. Exp. Biol. 187, 143-158.

Wassom, D.L. and Kelly, E.A.B. 1990. The role of the major histocompatibility complex in resistance to parasite infections. Crit. Rev. Immunol. 10, 31-52.

Westerfield, M. 1993. The zebrafish book. A guide for the laboratory use of zabrafish (*Brachydanio rerio*). Inst. Neurosc., University of Oregon Press, Oregon, USA.

Wiegertjes, G.F., Groeneveld, A. and van Muiswinkel, W.B. 1995a. Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). Vet. Immunol. Immunopathol. 47, 153-161.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1994. Divergent selection for antibody production in common carp (*Cyprinus carpio* L.). Anim. Genet. 25, 251-257.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1995b. Investigations into the ubiquitous nature of high or low immune responsiveness after divergent selection for antibody production in common carp (*Cyprinus carpio* L.). Vet. Immunol. Immunopathol., in press.

Wiegertjes, G.F., Stet, R.J.M. and van Muiswinkel, W.B. 1995c. Divergent selection for antibody production to produce standard carp (*Cyprinus carpio* L.) lines for the study of disease resistance in fish. Aquaculture, in press.

Zarnecki, A., Eide, D.M., Røed, K.H. and Rønningen, K. 1994. Immune traits as indicators for survival in Atlantic salmon (*Salmo salar*). In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph, Vol. XX, pp. 226-229.

Chapter 7

General discussion

The divergent selection for antibody production described in this thesis, using gynogenetic reproduction, resulted in the establishment of a number of F_1 hybrids of homozygous carp with different immune response types after immunization with the selection antigen (dinitrophenyl-keyholelimpet haemocyanin; DNP-KLH). Typically, these isogenic lines showed no within-line genetic variation, as assessed by DNA fingerprints, and a reduced variation in antibody production to DNP-KLH, compared with the base population. The ubiquitous nature of the differences in immune responsiveness was investigated using hapten-carrier complexes other than DNP-KLH. Furthermore, the resistance to a pathogen was examined, using a haemoflagellate parasite infection model. The characterization of the established isogenic carp lines, is described, and discussed, in chapter 6. Therefore, in the present chapter, genetic control mechanisms which could have a regulatory function in determining the differences between the high- and low-responder lines, will be discussed as likely candidates for further investigation. Furthermore, a number of miscellaneous observations that were made during the experiments, which could be of importance to the explanation of the outcome of the selection procedure, will be discussed. Finally, the selection model is set into the context of studies on genetic aspects of disease resistance in fish.

Inbred fish lines

Experience with induced gynogenetic reproduction via suppression of the first mitotic division, generating a variety of presumed homozygous offspring within one generation only, is rapidly increasing. The gynogenetic reproduction of such homozygous females generates fish lines without genetic variation, which may be highly suitable for biomedical research (Powers, 1989). However, practical limitations have also become clear. A number of reports have demonstrated a strong reduction in the reproductive capacity of gynogenetic homozygous females (Komen *et al.*, 1992a; Quillet, 1994; Rothbard, 1994; Johnstone and Stet, 1995), limiting the possibility to select for parameters other than reproductive capacity. The occurrence of functional XX males, within homozygous families, is highly convenient for the production of genetically-identical offspring via F_1 hybrids of homozygous carp (Komen *et al.*, 1992b). However, the establishment of homozygous fish lines, as well as future F_1 generations, depends upon the possibilities to maintain the homozygous genotypes. The androgenetic reproduction of the homozygous male genotype has recently become feasible for carp (Bongers *et al.*, 1994), but gynogenetic reproduction of the female homozygous genotype has been estimated to be successful for less than 10% of the animals (Komen, 1990). For this reason, we chose to preselect two individuals from a base population, one for high (No. 69), the other for low antibody (No. 85) production. It was shown that the high-and low-responder phenotypes were retained within the corresponding homozygous families (chapter 2), which

doubled the expected number of reproductive homozygous carp with high- or low-responder phenotypes. In addition, to investigate a genetic influence on female reproductive capacity, simultaneous to the selection for immune responsiveness, a divergent selection for egg quality was undertaken (Bongers *et al.*, in preparation). This allowed a selection for antibody production, while taking into account the negative effects of inbreeding on reproduction. It was concluded that enough genetic variation existed to select females with good egg quality, and as a consequence, recently, a number of homozygous carp lines have been successfully established, via gynogenetic reproduction of several females (G.F. Wiegertjes and A.B.J. Bongers, unpublished data, 1995).

Occasionally, we observed the occurrence of high numbers of diploid embryos when no treatment to induce female chromosome diploidization was applied (controls for haploidy). These unexpected diploid animals were mirror carp, *i.e.* they did not have the marker for paternal inheritance (scalation). A similar occurrence of spontaneous diploidization, for some common carp individuals, was recently shown to be heritable (Cherfas *et al.*, 1995). However, the cytological mechanism determining this event remained unclear. A number of underlying mechanisms could be responsible, such as elimination of meiosis, premeiotic mitosis or genome exclusion, which have all been reported to occur in unisexual fish species that reproduce naturally by gynogenesis (see chapter 1, page 23). Another possibility could be the incorporation of small amounts of paternal DNA, via microchromosomes possibly undamaged by the UV irradiation treatment of the sperm cells, as was recently shown to occur with sperm from related bisexual species in the natural gynogenetic reproduction of the Amazon molly (*Poecilia formosa*; Schartl *et al.*, 1995). Although we detected diploid mirror carp in the offspring of low-responder female No. 85, both the absence of triploid animals within the AbL family (flow cytometric analysis; G.F. Wiegertjes, unpublished data, 1994), and lack of within-line variation in fingerprints of F_1 hybrids of this family (chapter 6), suggested that this event had no influence on the presumed homozygous nature of this gynogenetic offspring. Spontaneous diploidization events were also observed for some selected gynogenetic females, within both families. Since these animals are presumed homozygous, spontaneous diploid offspring should be identical to the mother, independent from the cytological mechanism underlying the trait. Comparison of DNA fingerprints of such offspring, with that of induced gynogenetic offspring of the same mother, could possibly confirm this hypothesis. If the groups are genetically identical, this phenomenon could be used to produce large numbers of genetically-identical carp, to overcome the practical limitations set to the reproductive capacity of homozygous gynogenetic females.

The selection antigen

Traditionally, hapten-carrier complexes have been used to replace complex proteins, or even complete cells such as sheep erythrocytes, as antigen in studies on the mechanism of T-dependent antibody responses. A so-called carrier effect is observed when an animal is primed with a certain hapten-carrier complex, but challenged with the same hapten coupled to an unrelated carrier. No secondary response to the hapten will be observed under these circumstances. Although immunization against a hapten-carrier results in an antibody response to the hapten, as well as to determinants of the carrier molecule, most often only the anti-hapten response is measured. The current hypothesis for the hapten-carrier response is that the B cell recognizes the hapten with its immunoglobulin receptor and presents the processed peptides derived from the carrier, that can bind to 'self' major histocompatibility complex molecules, to T cells. It is not clear whether direct contact between the T and B cell is needed, or that the B cell may become responsive to T cell-derived cytokines. Although the above described model is based upon the knowledge of the mammalian immune system (Austyn and Wood, 1993), the hapten-carrier effect has since long been recognized to occur in fish (Yocum *et al.*, 1975). The model is assumed to be applicable to the fish immune system also (Vallejo *et al.*, 1992).

In our studies, we observed high anti-DNP titres but no detectable primary response to KLH, although after second immunization increased anti-KLH antibody titres were observed (G.F. Wiegertjes, unpublished data, 1995). Recent evidence has indicated that intramolecular-induced suppression, observed in response to hapten-carrier immunization of Atlantic salmon (*Salmo salar*), may be a distinct phenomenon more restricted to fish (Killie and Jørgensen, 1994). The response to immunodominant hapten determinants was found to be induced at the expense of the response to carrier determinants. Previously, in carp, Ambrosius and Frenzel (1972), and Avtalion and Milgrom (1976), also detected a lack of anti-carrier response. The latter authors found that in carp, but not in rabbits, heavily conjugated carrier molecules lost the ability to stimulate B cells, but not the ability to stimulate T cells. Possibly, as postulated for Atlantic salmon (Killie and Jørgensen, 1995), natural anti-DNP activity in normal carp serum, which can be relatively high (chapter 2), can result in antigen-induced suppression (AIS). The authors argue that AIS in fish is non-specific and represents a possible explanation for the functional restrictiveness of the antibody repertoire generally observed in fish (Du Pasquier, 1982). The possibility that a different degree of AIS in the high- and low-responder lines may be (partly) responsible for the different immune response types, warrants an investigation of the anti-KLH antibody titres of these carp. No differences in pre-immune anti-DNP titres were detected between the carp lines, however. Also, the carrier-dependency of the anti- DNP-KLH immune response (chapters 5, 6), suggests the involvement of T cells.

Detection of immune response gene control

We observed that low-responder carp did not exhibit an absence of responsiveness to DNP-KLH, but rather a lower antibody production relative to that of high-responder carp. Immune response (Ir) gene control of all-or-none responsiveness is mostly seen after immunization with relatively simple amino acid polymers. Although the same phenomenon has been found to apply for more complex antigens, high or low responsiveness rather than all-or-none is most frequently observed (see general introduction, page 12). Kojima *et al.* (1988) investigated the mechanisms determining Ir gene-controlled high or low responsiveness in the mouse, by using limiting dilution of T cell lines from high x low responders to sperm whale myoglobulin maintained on high or low responder antigen presenting cells. They concluded that low responsiveness was due primarily to the failure to respond to a single immunodominant epitope. Even though a number of immunodominant epitopes could be recognized by low responders, likely the response to these epitopes could never reach the level of response to the immunodominant epitope.

The use of well-defined antigenic determinants to select for immune responsiveness likely increased the detection level of a presumed genetic control. Our choice for a hapten-carrier complex as selection antigen was based on the established correlation of the immune response to hapten-carriers with the major histocompatibility complex (Mhc) in mammals (Klein, 1986). The results indicated that, for a number of reasons, the selection for high and low immune responsiveness to DNP-KLH has been influenced by the carp Mhc. The outcome of the selection was likely carrier-dependent (chapters 5, 6). Similarly, in the mouse, the genetic control to low doses of dinitrophenyl conjugates of ovomucoid and bovine γ-globulin was shown to be linked to the *H-2* complex, and was solely dependent on the protein carrier (Freed *et al.*, 1976). Particular F_1 hybrids of homozygous carp could be high responders to DNP-KLH but low responders to DNP-HSA, whereas others were low responders to DNP-KLH but high responders to DNP-HSA (chapter 6). Similar observations in inbred guinea pigs demonstrated that the anti-hapten response was linked to an Ir gene involved in controlling responsiveness to the carrier molecule (Green *et al.*, 1972).

Although the organization of the genes within the teleost Mhc is still unclear, many new sequences for all classes of the Mhc have been described since the cloning of gene fragments from a putative teleost Mhc was reported (Hashimoto *et al.*, 1990). It is now known that teleost Mhc proteins contain many conserved features and that the genes encoding them are generally similar in structure to the warm-blooded vertebrate Mhc genes (Dixon *et al.*, 1995). We examined the parental fish that were used to constitute the base population for previously described Mhc class II β-chain genes. Early/high responsiveness, as defined in the base population, was shown to be associated with a relative increase in frequency of *Cyca-*

*DAB1*01/DAB2*01* genes, whereas late/low responsiveness was associated with an increased frequency of *Cyca-DAB3*01/DAB4*01* genes (chapter 6). The aberrant splice site sequence (Jacob and Gallinaro, 1989) characterizing the latter Mhc genes, can result in lower expression levels of these Mhc molecules, which may have contributed to low responsiveness. The female parent of the AbH family (No. 69) was taken from the early/high, the parent of the AbL family (No. 85) from the late/low responder group. Therefore, these two parents similarly differed for *Cyca-DAB*. Consequently, all high-responder F_1 hybrids carried the *Cyca-DAB1*01/DAB2*01* genes, whereas all low-responder F_1 hybrids carried the *Cyca-DAB3*01/DAB4*01* genes. The high antibody production of the AbH x AbL crosses demonstrated that high responsiveness to DNP-KLH was dominant, maybe because these fishes carried the *Cyca-DAB1*01/DAB2*01* genes.

The results of the infection experiment with *Trypanoplasma borreli*, as described and discussed in chapter 6, suggested that the two parents selected to constitute the AbH and AbL families were different for at least one gene with a major influence on resistance to the parasite. Particular Mhc haplotypes (such as $I\text{-}E^k$) have been linked to increased susceptibility of mice to related parasites such as *Trypanosoma cruzi* (Powell and Wassom, 1993). The aberrant splice site sequence of the Mhc genes found in the low-responders, if indeed resulting in lower expression levels on the cell surface, could have had an effect on the presentation of *T. borreli*-derived antigenic fragments. Of course, other (major) genes with an effect on immune responsiveness, may have co-segregated with the Mhc, confounding the genetic effects on the differences between the two families. Recently, however, the genes controlling quantitative antibody production in Biozzi mice, were mapped using microsatellite markers, and confirmed the important role for the Mhc, next to the immunoglobulin locus and other polymorphic loci (Puel *et al.*, 1995).

Non-Mhc genes

In general, immune responsiveness is a quantitative trait under influence of several genes, some of which are in the Mhc, which has a central role in the acquired immune response. The outcome of the selection by Biozzi *et al.* (1979, 1984) was that high macrophage activity was for a large part responsible for low antibody synthesis, whereas low macrophage activity favoured high antibody responsiveness (general introduction, page 8). Dockrell *et al.* (1985) measured a number of macrophage functions, in two substrains that were separated from the original breeding stock. The results supported but sometimes also seemed to contradict these conclusions. The production of reactive oxygen intermediates was always higher in high-responder than in low-responder mice, although higher titres of tumour necrosis factor could be induced in low-responder mice. Possibly, because of genetic drift, these lines differed in

some aspects from the original breeding stock, as observed for the *H-2* complex (Liu *et al.*, 1993). We are currently examining possible differences in the capacity of head kidney macrophages and neutrophilic granulocytes from high- and low-responder carp to be stimulated by phorbol esters *in vitro* (Verburg-van Kemenade *et al.*, 1994).

In contrast, the differences between high- and low-responder mice selected for antibody production against somatic antigen of *Salmonella*, instead of sheep erythrocytes, were not based upon the modification of antigen processing and presentation at the macrophage level, but probably due to a less efficient response of T helper cells to immunogenic challenge in the low-responder mice (Reis *et al.*, 1992). Examination of the role of T (helper) cells in the immune response to DNP-KLH, and of possible differences in function between our high- and low-responder carp, is seriously hampered by the lack of specific markers characterizing T cell populations in fish. However, the recent isolation and characterization of the T cell receptor genes in rainbow trout (*Oncorhynchus mykiss*, Partula *et al.*, 1994), will most likely accelerate further investigations into T cell function within the teleost immune system.

We have used ELISA optical density values for the selection of high- and low-responder carp. It has been suggested that ELISA using the direct absorbance approach measures antibody affinity, whereas end-point titration measures antibody concentration (Steward and Lew, 1985). The Biozzi high and low responder mice produced antibody of the same affinity (Katz and Steward, 1976), but divergent breeding experiments have indicated a genetic control of antibody affinity in the mouse, independent from antibody levels and the Mhc (Steward *et al.*, 1979). There are a number of reasons to assume that the differences in antibody response between our carp lines are based on antibody concentration rather than on differences in affinity. First, the isogenic F_1 hybrids were also shown to differ in ELISA end-point titrations. Secondly, the use of high epitope density ($DNP_{44}BSA$) in the ELISA minimized the effects of affinity on the antibody concentration (Steward and Lew, 1985). Thirdly, carp have been shown to produce preferentially IgM antibodies of high functional affinity after injection of hapten-carrier antigen ($DNP_{10}HSA$; Fiebig *et al.*, 1977). To confirm this hypothesis, we are currently using an ELISA-based technique for the analysis of affinity (Nieto *et al.*, 1984), which has recently been adjusted for fish antibodies (Kaattari and Shapiro, 1994). In this assay, only the antibodies with the highest affinities bind to the minimum amount of coated antigen.

In conclusion, the consequences of (divergent) selection for immune responsiveness are generally difficult to predict, and the nature of the immunogenetic changes can be best described in retrospect. The choice of the selection antigen, as well as the immunization procedure, have been shown to be two major factors determining the gene interaction operating in many selective breeding experiments (Ibanez *et al.*, 1988).

Resistance to pathogens

In this thesis, we have described the selection of isogenic carp lines with a predetermined difference in their antibody response to a simple antigen. It may be difficult to extend the genetic regulation of antibody production to DNP-KLH, to similar differences in the immune response to complex antigens such as pathogens. We have investigated possible differences in disease resistance between the high- and low-responder carp, using the parasite *Trypanoplasma borreli*, because protective antibody was shown to play an important role in the resistance to the haemoflagellate (chapter 4). Also, in the same chapter, particular carp lines were described that were not able to produce specific antibodies to both DNP-KLH and *T. borreli*. These observations were confirmed in the high- and low-responder F_1 hybrids (chapter 6). Although disease models based on parasite infections can be complex in nature, the replacement of the natural (leech) vector by injection simplified the experimental model. The possibility to introduce carefully established numbers of infective parasites allowed manipulation of the infection dose. There are two other disease models available for infection of carp. *Rhabdovirus carpio* is a viral pathogen for which an experimental challenge methodology has been developed (Haenen and Davidse, 1993). Little is known however, on the immune defense mechanisms against this particular pathogen. More information is available on the immune response to *Aeromonas salmonicida* subspecies *nova*, for which laboratory challenge is an established technique (Daly *et al.*, 1994). The protective immune response to this bacterium is still under debate (Verburg-van Kemenade *et al.*, 1995), but so far, specific antibodies have not been shown effective in controlling infection.

Carp with a genetically-determined low antibody response (AbL family) were more susceptible to infection with *Trypanoplasma borreli* than carp from the AbH family with a high antibody response. In general, the low-responder carp failed to produce specific antibodies upon immunization with γ-irradiated trypanoplasms, while high-responder carp did (chapter 6). Passive immunization could (partially) protect naive carp from subsequent lethal infection (chapter 4), whereas carp that survive an infection with *T. borreli* have been shown resistant to re-infection (Jones *et al.*, 1993). Although the recent demonstration that *in vitro* exposure of carp leucocytes to *T. borreli* strongly inhibited their proliferation, indicating immunodepression by these parasites (Jones *et al.*, 1995), clearly suggests a complex picture, these observations suggest that, indeed, antibody production could be important for protection.

Vaccination trials in carp with lysed, or formalin-treated trypanoplasms failed to induce specific antibody production and protection (G.F. Wiegertjes, unpublished data, 1993), but immunization with γ-irradiated *T. borreli* resulted in increased antibody levels. This suggests that (protective?) antibody responses may be best initiated by immunization with attenuated parasites, allowing the fish to respond to critical antigenic determinants on the membrane of

the parasite. Similarly, protection against *Cryptobia salmositica*, a related haemoflagellate parasite of salmonid fish, could be obtained by immunization of juvenile rainbow trout (*Oncorhynchus mykiss*) with live parasites attenuated by continuous *in vitro* culture (Sitja-Bobadilla and Woo, 1994). The titre of complement-fixing antibodies, able to lyse the parasite *in vitro*, was higher in vaccinated and challenged fish than in unvaccinated and challenged ones. Interestingly, innate resistance of particular strains of brook charr (*Salvelinus fontinalis*), was controlled by a single dominant gene, possibly with an effect on complement activation (Woo, 1995). Possibly, in carp infected with *T. borreli*, complement-mediated lysis could be important in the acute phase, whereas sensitization of macrophages could be more important during the chronic stage of infection, such as previously suggested by Jones and Woo (1987) for *C. salmositica* infection of rainbow trout.

Conclusions and future prospects

We have addressed the complex problem of establishing immunogenetic factors that can be important for disease resistance in fish by developing both an animal model and a disease model. We were able to produce isogenic carp lines with predetermined responsiveness in two generations only, which should allow for a repeated establishment of other isogenic carp lines with a variety of (predetermined) immune characteristics. The high fecundity of carp offers the possibility for elaborate segregation studies of polymorphic traits involved in, for instance, disease resistance. Clear differences existed between the high- and low-responder lines with respect to their Mhc genes, and with respect to resistance to parasite infection. However, the exact nature of the immunogenetic differences between the high- and low-responder lines needs further investigation. As outlined in the general introduction (chapter 1, page 5), a first phase of a long-term strategy to investigate the genetic control of disease resistance can consist of the development of a set of suitable parameters to obtain clear-cut differences between inbred lines. We have shown that both antibody production to DNP-KLH and antibody production to *T. borreli* correlated with differences in resistance to the parasite. Parasite-resistance was inherited in a dominant fashion in AbH x AbL F_1 hybrids. Now, the next phase of the strategy can consist of a screening for Mendelian patterns of inheritance in F_2 and backcrosses to susceptible and resistant parents. Analogous to the mapping of genes controlling the quantitative antibody production in Biozzi mice (Puel *et al.*, 1995), mapping of genes controlling the response to DNP-KLH in our carp lines may become feasible in the future with the development of polymorphic microsatellite markers for carp (Komen *et al.*, in preparation). Furthermore, haploid gynogenesis can assist classical linkage studies, since (female) haploids are direct reflectors of the maternal haplotypes or their recombinants (Lie *et al.*, 1994).

In combination with information on genes and mechanisms described for other animal species, isogenic lines may aid substantially to the immunogenetic knowledge of disease resistance in fish. Of special interest are the striking similarities between the effects of the single dominant gene *Nramp*, which modulates the growth rate of intracellular pathogens of mouse macrophages during early infection (general introduction, page 14), and cattle resistance to *Brucella* infections as well as patterns of resistance of chickens to infection with *Salmonella* (Malo *et al.*, 1994). Also, high sequence similarity between a genomic DNA fragment in perch (*Perca fluviatilis*), and the *Mx* gene in the mouse (Staeheli *et al.*, 1989), which controls the susceptibility to infection with influenza virus, has been observed. This candidate gene approach to resolve the mutational basis of inherited diseases may selectively reduce the sensitivity for diseases of livestock (Womack, 1992).

References

Ambrosius, H. and Frenzel, E-M. 1972. Anti-DNP antibodies in carps and tortoises. Immunochemistry 9, 65-71.

Austyn, J.M. and Wood, K.J. 1993. Principles of cellular and molecular immunology. Oxford University Press, Oxford, UK.

Avtalion, R.R. and Milgrom, L. 1976. Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. I. Influence of hapten density on the immunological and serological properties of penicilloyl-carrier conjugates. Immunology 31, 589-594.

Biozzi, G., Mouton, D., Sant'Anna, O.A., Passos, H.C., Gennari, M., Reis, M.H., Ferreira, V.C.A., Heumann, A.M., Bouthillier, Y., Ibanez, O.M., Stiffel, C. and Siqueira, M. 1979. Genetics of immunoresponsiveness to natural antigens in the mouse. Curr. Top. Microbiol. Immunol. 85, 31-98.

Biozzi, G., Mouton, D., Stiffel, C. and Bouthillier, Y. 1984. A major role of the macrophage in quantitative genetic regulation of immunoresponsiveness and antiinfectious immunity. Advances in Immunol. 36, 189-233.

Bongers, A.B.J., in 't Veld, E.P.C., Abo-Hashema, K., Bremmer, I.M., Eding, E.H., Komen, J. and Richter, C.J.J. 1994. Androgenesis in common carp (*Cyprinus carpio* L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture 122, 119-132.

Cherfas, N., Gomelsky, B., Ben-Dom, N. and Hulata, G. 1995. Evidence for the heritable nature of spontaneous diploidization in common carp, *Cyprinus carpio* L., eggs. Aquacult. Research 26, 289-292.

Daly, J.G., Wiegertjes, G.F. and van Muiswinkel, W.B. 1994. Protection against carp erythrodermatitis following bath or subcutaneous exposure to sublethal numbers of virulent *Aeromonas salmonicida* subsp. *nova*. J. Fish Dis. 17, 67-75.

Dixon, B., van Erp, S.H.M., Rodrigues, P.N.S., Egberts, E. and Stet, R.J.M. 1995. Fish major histocompatibility complex genes: an expansion. Dev. Comp. Immunol. 19, 109-133.

Dockrell, H.M., Taverne, J., Lelchuk, R., Depledge, P., Brown, I.N. and Playfair, J.H.L. 1985. Macrophage functions in Biozzi mice. Immunology 55, 501-509.

Du Pasquier, L. 1982. Antibody diversity in lower vertebrates - why is it so restricted? Nature 296, 311-313.

Fiebig, H., Gruhn, R. and Ambrosius, H. 1977. Studies on the control of IgM antibody synthesis III. Preferential formation of anti-DNP-antibodies of high functional affinity in the course of the immune response in carp. Immunochemistry 14, 721-726.

Freed, J.H., Deak, B.D. and McDevitt, H.O. 1976. Mapping of the genetic control of murine response to low doses of dinitrophenyl conjugates of ovomucoid and bovine γ-globulin. J. Immunol. 117, 1514-1518.

Green, I., Paul, W.E. and Benacerraf, B. 1972. Histocompatibility-linked genetic control of the immune response to hapten guinea pig albumin conjugates in inbred guinea pigs. J. Immunol. 109, 457-463.

Haenen, O.L.M. and Davidse, A. 1993. Comparative pathogenicity of two strains of pike fry rhabdovirus and spring viraemia of carp virus for young roach, common carp, grass carp and rainbow trout. Dis. Aquat. Org. 15, 87-92.

Hashimoto, K., Nakanishi, T. and Kurosawa, Y. 1990. Isolation of carp genes encoding major histocompatibility complex genes. Proc. Natl. Acad. Sc. USA 87, 6863-6867.

Ibanez, O.M., Mouton, D., Oliveira, S.L., Fihlo, O.G.R., Piatti, R.M., Sant'Anna, O.A., Massa, S., Biozzi, G. and Siqueira, M. 1988. Polygenic control of quantitative antibody responsiveness: restrictions of the multispecific effect related to the selection antigen. Immunogenetics 28, 6-12.

Jacob, M. and Gallinaro, H. 1989. The 5' splice site: phylogenetic evolution and variable geometry of association with U1RNA. Nucl. Acids Res. 17, 459-472.

Johnstone, R. and Stet, R.J.M. 1995. The production of gynogenetic Atlantic salmon, *Salmo salar* L. T.A.G. 90, 819-826.

Jones, S.R.M. and Woo, P.T.K. 1987. The immune response of rainbow trout, *Salmo gairdneri* Richardson, to the haemoflagellate, *Cryptobia salmositica* Katz, 1951. J. Fish Dis. 10, 395-402.

Jones, S.R.M., Palmen, M. and van Muiswinkel, W.B. 1993. Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. Vet. Immunol. Immunopathol. 36, 369-378.

Jones, S.R.M., Wiegertjes, G.F. and van Muiswinkel, W.B. 1995. Modulation of carp (*Cyprinus carpio* L.) cellular immune function *in vitro* caused by the blood parasite *Trypanoplasma borreli* Laveran & Mesnil. Fish Shellf. Immunol. 5, 381-383.

Kaattari, S.L. and Shapiro, D.A. 1994. Determination of serum antibody affinity distributions using ELISA-based technology. In: Techniques in fish immunology III (eds: Stolen, J.S., Fletcher, T.C., Rowley, A.F., Zelikoff, J.T., Kaattari, S.L. and Smith, S.A.). SOS Publ., Fair Haven, NJ, USA, pp. 85-97.

Katz, F.E. and Steward, M.W. 1976. Studies on the genetic control of antibody affinity. The independent control of antibody levels and affinity in Biozzi mice. J. Immunol. 117, 477-479.

Killie, J-E.A. and Jørgensen, T.Ø. 1994. Immunoregulation in fish I: intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L.). Dev. Comp. Immunol. 18, 123-136.

Killie, J-E.A. and Jørgensen, T.Ø. 1995. Immunoregulation in fish II: intermolecular-induced suppression of antibody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L.). Dev. Comp. Immunol. 19, in press.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley & Sons, New York.

Kojima, M., Cease, K.B., Buckenmeyer, G.K. and Berzofsky, J.A. 1988. Limiting dilution comparison of the repertoires of high and low responder Mhc-restricted T cells. J. Exp. Med. 167, 1100-1113.

Komen, J. 1990. Clones of common carp. New perspectives in fish research. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Komen, J., de Boer, P. and Richter, C.J.J. 1992a. Male sex-reversal in gynogenetic XX females of common carp (*Cyprinus carpio* L.) by a recessive mutation in a sex-determining gene. J. Hered. 83, 431-434.

Komen, J., Wiegertjes, G.F., van Ginneken, V.J.T., Eding, E.H. and Richter, C.J.J. 1992b. Gynogenesis in common carp (*Cyprinus carpio* L.) III. The effects of inbreeding on gonadal development of heterozygous and homozygous gynogenetic offspring. Aquaculture 104, 51-66.

Lie, Ø., Slettan, A., Lingaas, F., Olsaker, I., Hordvik, I. and Refstie, T. 1994. Haploid gynogenesis: a powerful strategy for linkage analysis in fish. Anim. Biotechn. 5, 33-45.

Liu, G.Y., Baker, D., Fairchild, S., Figueroa, F., Quartey-Papafio, R., Tone, M., Healy, D., Cooke, A., Turk, J.L. and Wraith, D.C. 1993. Complete characterization of the expressed immune response genes in Biozzi Ab/H mice: structural and functional identity between Ab/H and NOD *A* region molecules. Immunogenetics 37, 296-300.

Malo, D., Hu, J., Skamene, E. and Schurr, E. 1994. Population and molecular genetics of susceptibility to intracellular pathogens. Anim. Biotechn. 5, 173-182.

Nieto, A., Gaya, A., Jansa, M., Moreno, C. and Vives, J. 1984. Direct measurement of antibody affinity distribution by hapten inhibition enzyme immunoassay. Mol. Immunol. 21, 537-543.

Partula, S., Fellah, J.S., de Guerra, A. and Charlemagne, J. 1994. Identification of cDNA clones encoding the T-cell receptor β-chain in the rainbow trout (*Oncorhynchus mykiss*). C.R. Acad. Sci. Paris, France. Life Sci. 317, 765-770.

Powell, M.R. and Wassom, D.L. 1993. Host genetics and resistance to acute *Trypanosoma cruzi* infection in mice. I. Antibody isotype profiles. Parasite Immunol. 15, 215-221.

Powers, D.A. 1989. Fish as model systems. Science 246, 352-358.

Puel, A., Groot, P.C., Lathrop, M.G., Demant, P. and Mouton, D. 1995. Mapping of genes controlling quantitative antibody production in Biozzi mice. J. Immunol. 154, 5799-5805.

Quillet, E. 1994. Survival, growth and reproductive traits of mitotic gynogenetic rainbow trout females. Aquaculture 123, 223-236.

Reis, M.H., Ibanez, O.M., Cabrera, W.H., Ribeiro, O.G., Mouton, D., Siqueira, M. and Couderc, J. 1992. T-helper functions in lines of mice selected for high or low antibody production (Selection III): modulation by anti-$CD4^+$ monoclonal antibody. Immunology 75, 80-85.

Rothbard, S. 1994. Cloning of Nishiki-Goi, Japanese ornamental (Koi) carp. Bamidgeh 46, 171-181.

Schartl, M., Nanda, I., Schlupp, I., Wilde, B., Epplen, J.T., Schmid, M. and Parzefall, J. 1995. Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish. Nature 373, 68-71.

Sitja-Bobadilla, A. and Woo, P.T.K. 1994. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against the pathogenic haemoflagellate, *Cryptobia salmositica* Katz, and protection against cryptobiosis in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), inoculated with a live vaccin. J. Fish Dis. 17, 399-408.

Staeheli, P., Yu, Y-X., Grob, R. and Haller, O. 1989. A double-stranded RNA-inducible fish gene homologous to the murine influenza virus resistance gene *Mx*. Mol. Cell. Biol. 9, 3117-3121.

Steward, M.W. and Lew, A.M. 1985. The importance of antibody affinity in the performance of immunoassays for antibody. J. Immunol. Methods 78, 173-190.

Steward, M.W., Reinhardt, M.C. and Staines, N.A. 1979. The genetic control of antibody affinity. Evidence from breeding studies with mice selectively bred for either high or low affinity antibody production. Immunology 37, 697-703.

Vallejo, A.N., Miller, N.W. and Clem, L.W. 1992. Antigen processing and presentation in teleost immune responses. Ann. Rev. Immunol. 2, 73-89.

Verburg-van Kemenade, B.M.L., Groeneveld, A., van Rens, B.T.T.M. and Rombout, J.H.W.M. 1994. Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). J. Exp. Biol. 187, 143-158.

Verburg-van Kemenade, B.M.L., Daly, J.G., Groeneveld, A. and Wiegertjes, G.F. 1995. Multiple regulation of carp (*Cyprinus carpio* L.) macrophages and granulocytes by serum factors: influence of infection with atypical *Aeromonas salmonicida*. Vet. Immunol. Immunopathol., in press.

Womack, J.E. 1992. Molecular genetics arrives on the farm. Nature 360, 108-109.

Woo, P.T.K. 1995. Strategies against salmonid cryptobiosis. Bull. Soc. Can. Zool. 26, 50.

Yocum, D. Cuchens, M. and Clem, W. 1975. The hapten-carrier effect in teleost fish. J. Immunol. 114, 925-927.

Current methods to control diseases in livestock, including fish, consist of hygiene, vaccination, medication and eradication of affected animals. Improving genetic disease resistance may be another, preventive, measure against infectious diseases, which is attractive because of its prospects of prolonged protection. The ability of a host to resist infection with viral, bacterial and parasitic pathogens is strongly influenced by genetic factors. In the laboratory mouse, the majority of studies have either focused on the selection of inbred lines with a genetically determined response to particular pathogens, or on selective breeding for changes in immune responsiveness (**chapter 1**). The best known examples of the latter are certainly the divergent selections in the mouse for high and low antibody production to sheep red blood cells, initiated by Biozzi. Differences in macrophage metabolism showed a multispecific effect. The strong activity of the low-responder macrophages resulted in a relative increase in resistance to intracellular pathogens, and a decreased resistance to extracellular pathogens. Recently, two of the genes with an important regulating function in these lines were mapped to the major histocompatibility complex (Mhc) and immunoglobulin locus.

Livestock generally have relatively long generation intervals, which hampers selective breeding experiments. Also, the homozygous state of increasing numbers of genes, typical of inbred lines, can negatively affect fitness, which is the reason to avoid inbreeding. Instead, the most common approach for livestock is the search for associations between disease resistance and marker genes within the population. Markers can range from phenotypical features such as colour, to DNA polymorphisms of functional importance such as that of the Mhc. In fish, the importance of Atlantic salmon (*Salmo salar*) culture has led to an increasing interest in indirect selection criteria for disease resistance, such as (non)-specific immune parameters (**chapter 1**). Disease data have been analyzed for associations with immunogenetic markers, but so far, only few immune parameters, such as lysozyme, showed any promise as marker traits in selection for improved disease resistance.

More fundamental research on the genetic control of specific immune responsiveness is needed, before immune parameters can be exploited as markers in selection schemes for fish. The development of isogenic lines, or cloned lines, will contribute to a thorough analysis of the relationship(s) between immune parameters and disease resistance in fish. Some fish species, such as the Amazon molly (*Poecilia formosa*), produce cloned lines naturally, via so-called gynogenetic reproduction. Induced, or artificial, gynogenesis is essentially the same as the natural forms, but can be controlled in the laboratory. The male (irradiated) sperm cells are only required to start embryonic development, and have no genetic contribution. The haploid "embryo" is shock-treated to inhibit the first mitotic division. After this treatment, the embryo contains a duplicated set of maternal chromosomes and is therefore homozygous.

Subsequent gynogenetic reproduction will yield homozygous cloned lines, which can be used for experiments where genetic uniformity is required. The way to obtain genetic uniformity without increased environmental variation due to homozygosity, is to use the F_1 of a cross between two homozygous animals. Apparent technical problems have limited the production of cloned fish lines via artificial gynogenesis to only a few fish species, among which the common carp (*Cyprinus carpio* L.). The large size that carp can reach under laboratory circumstances makes this fish species particularly suitable for studies in cellular immunology. The high fecundity of carp will counteract the negative effects of induced gynogenesis on reproductive capacity.

The aim of the work described in this thesis is to investigate the possibility to select for immune responsiveness, and subsequently produce isogenic carp lines, via gynogenesis, that express the trait under selection. If possible, this would allow the repeated production of numerous isogenic fish lines, all selected for different immune parameters, that can be used for immunogenetic studies on disease resistance. As a model, we chose a defined antigenic determinant (dinitrophenyl: DNP), coupled to a carrier molecule (keyhole limpet haemocyanin: KLH) in combination with a reliable read-out system (enzyme-linked immunosorbent assay: ELISA), to divergently select carp for the magnitude of their primary antibody response. The use of simple antigenic determinants was assumed to increase the detection level of a genetic control of the immune response, and to facilitate the analysis of inherited differences that may have an effect on disease resistance.

Chapter 2 describes the immunization of a base population (n = 101), consisting of a single hybrid cross of 13-month-old female carp, with DNP-KLH. Subsequent measurement of the DNP-specific antibody response at 12 and 21 days after immunization, allowed the classification of a low number of individual females as early/high (10%) or late/low (13%) responders. Three individuals defined as early/high and three defined as late/low responding, were gynogenetically reproduced to obtain corresponding homozygous families within one generation. Upon immunization with DNP-KLH of the 6-month-old offspring, the antibody response was found to be significantly higher in the early/high responder homozygous families. These differences between the high and low responder families indicated a genetic influence on the antibody response (realized heritability h^2 = 0.37).

After the initial test of immune responsiveness at 6 months of age, we chose to continue the selection procedure with two families (**chapter 3**). Carp of the selected high-responder (n = 83) and low-responder families (n = 95) were immunized with DNP-KLH. Antibody production, at 12 months of age, was again shown to be consistent with the parental responder type (h^2 = 0.29). In contrast to the titres in the high-responder group, antibody titres in the low-responder family did not significantly increase after 21 days, in accordance with this

group's classification as late/low responders. A relative increase in frequency of early/high responders (45%) was seen in the high-responder family, compared with the low-responder family (11%).

Chapter 4 describes the development of a disease model, by examination of the antibody response to infection with *Trypanoplasma borreli*, a haemoflagellate parasite. Carp resistant to infection responded with a production of specific antibodies, but highly susceptible carp did not. This suggested an apparent relationship between susceptibility and the lack of specific antibody production. This relation was partially confirmed by the passive transfer of immunity with immune plasma. We were unable to demonstrate an antibody response to parasite-unrelated antigen (DNP-KLH) in two carp lines highly susceptible to the trypanoplasm (100% mortality), in contrast with outbred carp, the majority of which survived infection. This suggested that the low antibody response was not due to a poor health status, but that susceptible carp had a genetically predetermined low antibody response.

Two homozygous progenies, previously characterized as high or low responders to DNP-KLH, were immunized with T cell-dependent DNP-human serum albumin (HSA) and T cell-independent TNP-lipopolysaccharide (LPS) (**chapter 5**). In comparison with the anti- DNP-KLH response, antibody production to DNP-HSA was highly variable. The magnitude of antibody production to DNP-HSA was not different between the selected progenies, which suggested that the divergent selection had been carrier-specific. In contrast, immunization with T-independent TNP-LPS induced a very rapid response which differed between the high and low responders, and likely measured changes in the DNP-specific pool of B cells, caused by the selection. Individual homozygous carp were able to escape inbreeding depression and survive infection with *T. borreli*.

The possibility to reproduce both homozygous females and functional males with a high or a low antibody response, resulted in isogenic F_1 hybrid carp with presumptive differences in immune responsiveness (**chapter 6**). Typically, these isogenic lines showed no within-line variation in DNA fingerprints. It was found that, after immunization with DNP-KLH, immune sera from high-responder lines could be 1000-fold diluted, compared with immune sera from low responder carp, which could be approximately 100-fold diluted to obtain identical values in the ELISA. This confirmed previous suggestions for a genetic control of the antibody response to DNP-KLH. In general, the magnitude of antibody production to T cell-independent TNP-LPS corresponded with the responsiveness seen after DNP-KLH immunization. In contrast, immunization with T cell-dependent DNP-HSA induced opposite responsiveness, in some carp lines. The isogenic carp lines were infected with *T. borreli*. The genetic variation in susceptibility to the parasite was observed to be dependent upon the immune response type of the carp lines. This suggested that the two parents that were used to generate the high- and

low-responder homozygous families, differed for at least one gene with a major influence on resistance to *T. borreli*.

An attempt was made to use previously described Mhc genes, for a study on possible associations of Mhc class II β-chain polymorphism with the immune response types described for the base population (**chapter 6**). In most vertebrate species, including fish, Mhc molecules are highly polymorphic. The selection mechanism favouring extensive polymorphism is not yet clearly understood, but the most common view is that this phenomenon is related to significant influences of allelic Mhc molecules on the efficacy of the immune response and thereby on the resistance to pathogens. Although the Mhc may not be the only polymorphic system, it is by far the best described one, and instrumental to the outcome of an immune response. The parents of the base population differed with respect to their Mhc genes, which segregated in the base population. Early/high responsiveness was associated with a relative increase in frequency of *Cyca-DAB1*01/2*01* linked genes, whereas late/low responsiveness was associated with an increased frequency of *Cyca-DAB3*01/4*01* genes. The two parents that were selected to generate the high- and low-responder families similarly differed for *Cyca-DAB*. As a consequence, all low-responder F_1 hybrids carried the *Cyca-DAB3*01/4*01* genes, whereas all high-responders carried the *Cyca-DAB1*01/2*01* genes. In retrospect, these Mhc genes may have contributed to the observed differences in magnitude of immune responsiveness to DNP-KLH, and possibly *T. borreli*.

The successful divergent selection for antibody production described in this thesis suggests that a repeated production of isogenic carp lines selected for predetermined immune parameters is feasible (**chapter 7**). Such fish lines will certainly contribute to the immunogenetic knowledge of disease resistance in fish.

Samenvatting

Maatregelen ter bestrijding van ziekten in de veeteelt zijn verbetering van hygiëne, vaccinatie, medicatie en de eliminatie van besmette dieren. Selectie op genetische ziekteresistentie is een preventieve maatregel tegen infectieziekten, en is aantrekkelijk vanwege de verwachte langdurige bescherming. Genetische factoren hebben een sterke invloed op de bescherming van de gastheer tegen virale, bacteriële en parasitaire ziekteverwekkers. Het onderzoek aan de laboratorium muis heeft zich òf op selectie van inteeltlijnen, òf op selectieve veranderingen van de immuunrespons gericht (**hoofdstuk 1**). Een bekend voorbeeld van de laatste aanpak is de tweezijdige selectie op hoge en lage antilichaamproductie tegen schape rode bloedcellen. Verschillen in het metabolisme van de makrofagen veroorzaakten een meervoudig maar specifiek effect. De sterke activiteit van de makrofagen van laag reagerende dieren resulteerde in een relatieve toename in resistentie tegen intracellulaire -, en een relatieve afname in resistentie tegen extracellulaire ziekteverwekkers. Onlangs zijn twee van de genen met een belangrijke regulerende functie in deze muizelijnen in kaart gebracht als behorende tot het immunoglobuline locus en tot het hoofd-histocompatibiliteitscomplex (Engels: Major histocompatibility complex [Mhc]).

Over het algemeen hebben landbouwhuisdieren relatief lange generatie-intervallen, hetgeen selectie op ziekteresistentie bemoeilijkt. Tevens kan de homozygotie van een toenemend aantal genen, karakteristiek voor inteeltlijnen, de conditie verslechteren. Dit is een reden om inteelt te vermijden. Een aantrekkelijker aanpak bij landbouwhuisdieren is het zoeken naar associaties tussen ziekteresistentie en merker genen in de populatie. Merkers kunnen variëren van fenotypisch gemakkelijk herkenbare kenmerken zoals kleur, tot DNA polymorfismen met een functionele betekenis zoals dat van het Mhc. Voor wat vissen betreft, heeft het commerciële belang van de kweek van Atlantische zalm (*Salmo salar*) geleid tot een toename van onderzoek naar indirecte selectie criteria voor ziekteresistentie, zoals (a)specifieke immuunparameters (**hoofdstuk 1**). Tot nu toe blijken slechts enkele immuunparameters, zoals de produktie van lysozym, in aanmerking te komen als merker voor selectie op verbeterde ziekteresistentie.

Er is behoefte aan meer fundamentele kennis van de genetische invloeden op de specifieke immuunrespons, voordat deze gebruikt kan worden als merker in selectie programma's voor vis. De ontwikkeling van isogene lijnen, en van kloonlijnen, zal zeker een bijdrage leveren aan de kennis van relaties tussen immuunparameters en ziekteresistentie in vis. Sommige vissoorten, zoals de mollie (*Poecilia formosa*) uit de Amazone, produceren van nature kloonlijnen via gynogenetische voortplanting. Gynogenese kan in het laboratorium nagebootst worden. De mannelijke sperma cellen zijn nodig om de embryonale ontwikkeling te induceren en leveren geen genetische bijdrage doordat het DNA reeds voor bevruchting door bestraling uitgeschakeld wordt. Het haploïde 'embryo' wordt behandeld met een schok om de

eerste mitotische deling te verhinderen. Na deze behandeling bevat het embryo een dubbele set maternale chromosomen en is dus homozygoot. Een daaropvolgende gynogenetische voortplanting van deze individuen zal uitmonden in homozygote kloonlijnen, die gebruikt kunnen worden in die experimenten waarvoor genetische uniformiteit nodig is. Dé manier om genetische uniformiteit te verkrijgen zonder dat de omgevingsvariatie toeneemt door homozygotie (Engels: developmental instability), is een F_1 te gebruiken van een kruising tussen twee homozygote dieren. De produktie van gekloonde visselijnen is beperkt tot slechts enkele soorten, waaronder de karper (*Cyprinus carpio* L.). De aanzienlijke grootte die de karper kan bereiken in gevangenschap maakt de soort bijzonder geschikt voor wetenschappelijk onderzoek. De hoge fekunditeit compenseert de hoge mortaliteit die geïnduceerde gynogenese met zich meebrengt.

De doelstelling van het werk beschreven in dit proefschrift is de mogelijkheid tot selectie op de immuunrespons te onderzoeken. Geprobeerd is om, met behulp van gynogenese, isogene lijnen van de karper te ontwikkelen met vooraf bepaalde immunologische eigenschappen, geschikt voor immunogenetisch onderzoek naar ziekteresisentie. Als model hebben wij gekozen voor een gedefinieerde antigene determinant (Engels: dinitrophenyl: DNP) gekoppeld aan een drager eiwit (Engels: keyholelimpet haemocyanin: KLH) gecombineerd met een betrouwbaar meetsysteem (Engels: enzyme-linked immunosorbent assay: ELISA), om een tweezijdige selectie op de hoogte van de primaire antilichaamrespons bij de karper uit te voeren. We hebben aangenomen dat het gebruik van simpele antigene determinanten het aantonen van een genetische invloed op de immuunrespons zou vereenvoudigen, en dat het de analyse van overerfbare verschillen met een mogelijk effect op ziekteresistentie zou vergemakkelijken.

In **hoofdstuk 2** wordt de immunizatie met DNP-KLH beschreven, van een basispopulatie (n = 101) bestaande uit vrouwelijke karpers van 13 maand oud, uit een hybride kruising tussen twee dieren met een verschillende afstamming. De daaropvolgende meting van de DNP-specifieke antilichaamrespons op 12 en 21 dagen na immunizatie, resulteerde in een rangschikking van een beperkt aantal vrouwelijke individuen van vroeg/hoog reagerende (10%) of van laat/laag reagerende dieren (13%). Drie als vroeg/hoog, en drie als laat/laag gerangschikte individuen, werden gynogenetisch voortgeplant om binnen één generatie homozygote families met een vergelijkbare immuunrespons te krijgen. Na immunizatie met DNP-KLH van de 6 maand oude nakomelingen bleek de antilichaam-respons significant hoger in de vroeg/hoog reagerende homozygote families. Deze verschillen tussen de hoog en laag reagerende families wezen op een genetische invloed op de antilichaamproduktie (gerealiseerde erfelijkheidsgraad h^2 = 0.37).

Na de aanvankelijke test van de immuunrespons op een leeftijd van 6 maanden, kozen we voor een continuering van de selectie met twee families (**hoofdstuk 3**). Karpers van geselecteerde families met een hoge respons (n = 83), en met een lage respons (n = 95), werden geïmmuniseerd met DNP-KLH. De antilichaamproduktie op 12 maanden leeftijd was wederom vergelijkbaar met die van de ouders (h^2 = 0.29). In tegenstelling tot de titers van de hoog reagerende groep, vertoonden de antilichaam titers van de laag reagerende familie geen significante verhoging meer na 21 dagen, hetgeen in overeenstemming was met de rangschikking van deze groep als laat/laag reagerend. Er was een relatieve toename van de frequentie van vroeg/hoog reagerende dieren (45%) in de als hoog reagerend geclassificeerde familie, vergeleken met de frequentie in de als laag reagerend gekarakteriseerde familie (11%).

In **hoofdstuk 4** wordt de ontwikkeling van een ziektemodel beschreven, op basis van een onderzoek naar de respons op infectie met *Trypanoplasma borreli*, een parasitaire bloedflagellaat. Karpers resistent tegen infectie reageerden met een produktie van specifieke antilichamen, in tegenstelling tot sterk gevoelige karpers die geen antichamen produceerden. Dit veronderstelde een significante relatie tussen gevoeligheid en afwezigheid van specifieke antilichaamproduktie. Deze relatie werd gedeeltelijk bevestigd via passieve immunizatie met immuun plasma. In tegenstelling tot uitgeteelde karpers, waarvan de meerderheid de infectie overleefde, konden we in twee karperlijnen sterk gevoelig voor de trypanoplast (100% mortaliteit) geen antilichaamrespons aantonen tegen een antigeen dat niet gerelateerd was aan de parasiet (DNP-KLH). Dit zou er op kunnen wijzen dat de lage antilichaamrespons niet geheel veroorzaakt werd door een slechte conditie, maar dat de gevoelige karpers een genetisch bepaalde lage antilichaamrespons hadden.

Twee homozygote families, eerder gekarakteriseerd als hoog of laag reagerend op DNP-KLH, werden geïmmuniseerd met T cel afhankelijk DNP-humaan serum albumine (HSA) en T cel onafhankelijk TNP-lipopolysaccharide (LPS) (**hoofdstuk 5**). De hoogte van de antilichaamproduktie tegen DNP-HSA was sterk variabel in vergelijking tot de anti-DNP-KLH respons. De hoogte van de antilichaamproduktie tegen DNP-HSA verschilde niet tussen de geselecteerde families, hetgeen leidde tot de veronderstelling dat de tweezijdige selectie drager-specifiek moet zijn geweest. Echter, immunizatie met T cel onafhankelijk TNP-LPS riep een snelle respons op, die verschilde tussen de hoog en laag reagerende dieren. Deze reacties zouden verklaard kunnen worden door selectie in de verzameling DNP-specifieke B cellen. Na infectie bleek dat individuele homozygote karpers aan inteeltdepressie kunnen ontsnappen en een infectie met *T. borreli* kunnen overleven.

Homozygote vrouwtjes èn vrouwtjes die functioneren als mannetjes, met een hoge of lage antilichaamrespons, konden voortgeplant worden, hetgeen resulteerde in isogene F_1 hybride karpers met een verondersteld verschil in immuunreactiviteit (**hoofdstuk 6**). Het was

kenmerkend dat deze isogene lijnen geen binnen-lijn variatie in DNA-fingerprints (Engels) vertoonden. Immuun sera, verkregen na immmunizatie met DNP-KLH van hoog reagerende lijnen, konden 1000-voudig verdund worden, terwijl immuun sera van laag reagerende karpers ongeveer 100 keer verdund konden worden om vergelijkbare optische dichtheden te verkrijgen in de ELISA. Dit bevestigde het vermoeden dat de antilichaamrespons tegen DNP-KLH genetisch bepaald is. Over het algemeen was de hoogte van de antilichaamproduktie tegen T cel onafhankelijk TNP-LPS vergelijkbaar met de reactie tegen DNP-KLH. Immunizatie met T cel afhankelijk DNP-HSA wekte echter een tegengestelde respons op in sommige karperlijnen.

De isogene karperlijnen werden geïnfecteerd met *T. borreli*. De genetische variatie in gevoeligheid voor de parasiet bleek afhankelijk van het immuunrespons type van de karperlijnen. Dit leidt tot de veronderstelling dat de twee ouders gebruikt voor de produktie van de hoog en laag reagerende homozygote families verschillend zijn geweest voor tenminste één gen met een grote invloed op resistentie tegen *T. borreli*.

We hebben getracht eerder beschreven Mhc genen te gebruiken voor een studie naar een mogelijke associatie tussen Mhc β-keten polymorfisme en de immuunrespons typen beschreven voor de basispopulatie (**hoofdstuk 6**). Mhc moleculen zijn sterk polymorf in de meeste gewervelde dieren, waaronder de beenvissen. Het selectie mechanisme dat dit polymorfisme veroorzaakt is nog grotendeels onbekend. De meest gangbare mening is dat dit fenomeen gerelateerd is aan invloeden van allelische Mhc moleculen op de effectiviteit van de immuunrespons en daardoor op de resistentie tegen parasieten. De ouders van de basispopulatie verschilden met betrekking tot hun Mhc genen, welke segregeerden in de basispopulatie. Een vroeg/hoge immuunrespons kon worden gerelateerd aan een relatieve toename in de frequentie van *Cyca-DAB1*01/2*01* gekoppelde genen, terwijl een laat/lage immuunrespons gerelateerd was aan een toegenomen frequentie van *Cyca-DAB3*01/4*01* genen. De twee ouders geselecteerd voor de produktie van de hoog en laag reagerende homozygote families verschilden vergelijkbaar voor *Cyca-DAB*. Dientengevolge hadden alle laag reagerende F_1 hybriden de *Cyca-DAB3*01/4*01* genen, terwijl alle hoog reagerende dieren de *Cyca-DAB1*01/2*01* genen bezaten. Terugblikkend kan dus gesteld worden dat deze Mhc genen mogelijk bijgedragen hebben aan de waargenomen verschillen in de hoogte van de immuunreactie tegen DNP-KLH, en mogelijk ook tegen *T. borreli*.

De geslaagde tweezijdige selectie op antilichaamproduktie zoals beschreven in dit proefschrift veronderstelt dat een herhaalde produktie van isogene karperlijnen, geselecteerd op vooraf bepaalde immuunparameters, mogelijk is (**hoofdstuk 7**). Zulke isogene visselijnen zullen zeker bijdragen tot de immunogenetische kennis van de ziekteresistentie bij vissen.

Curriculum vitae

Geert Frits Wiegertjes werd geboren op 20 mei 1963 in Hoogezand-Sappemeer. Na het behalen van zijn diploma Atheneum-B aan het Hertog Jan college te Valkenswaard, begon hij in 1982 met zijn studie Zoötechniek aan de toenmalige Landbouwhogeschool te Wageningen. In september 1988 sloot hij zijn studie (afstudeervakken Celbiologie & Immunologie en Visteelt) af. Van mei 1989 tot december 1990 vervulde hij zijn vervangende dienstplicht bij de vakgroep Experimentele Diermorfologie en Celbiologie van de Landbouwuniversiteit te Wageningen, waar hij in december 1990 een functie als universitair docent (in deeltijd) kreeg. In november 1993 kreeg hij een aanvullende 3-jarige beurs van de Stichting voor de Technische Wetenschappen (SLW-STW) om zijn promotieonderzoek af te ronden met het thans voor u liggende resultaat.