Stellingen

1. Het schatten van de herhaalbaarheid om een bovengrens van de erfelijkheidsgraad te verkrijgen werkt niet bij complexe kenmerken zoals de stressrespons bij de karper.
   - dit proefschrift

2. Een koudeschok van 25 naar 16°C is een milde stressor voor de karper.
   - dit proefschrift

3. Sociale interacties hebben een grote invloed op de individuele stressrespons.

4. Selectie op cortisolrespons leidt niet tot verbetering van de algemene ziekteresistentie.

5. De lengte van de stressrespons is een betere maat voor het schadelijke effect van stress dan de hoogte van die respons.

6. Androgenetische nakomelingen zijn wel geschikt voor het vinden van associaties tussen merkers en QTL's, maar niet voor het vinden van daadwerkelijke genen.
   - Sakamoto et al., 2000. Genetics 155, 1331 – 1345

7. Reistijd is werktijd, aangezien de beste ideeën vaak ontstaan tijdens het reizen van en naar de werkplek.
   - Naar aanleiding van artikel Volkskrant, 30 oktober 2000

8. Het totstandkomen van één Europa leidt tot afname van de "biodiversiteit".

Stellingen behorende bij het proefschrift:

"Selective breeding for stress response in common carp (*Cyprinus carpio* L.) using androgenesis".

Michael Tanck

Wageningen, 20 december 2000

Selective breeding for stress response in common carp (*Cyprinus carpio* L.) using androgenesis

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Selective breeding for stress response in common carp (*Cyprinus carpio* L.) using androgenesis

Michael Tanck

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Table of contents

Chapter 1

General introduction

1 Scope of the thesis

Worldwide, the culture of fish and shellfish is expanding rapidly to satisfy the increasing demand for fish and shellfish products (Gjedrem, 1998). The total world production of fish, crustaceans and molluscs increased from 13.0 million metric tons in 1990 to 30.8 million metric tons in 1998. Two-thirds (20.8 metric tons) of the total production in 1998 were produced in China and carp, barbel and other cyprinids were the most popular species at 14 metric tons. The estimated value of the total production in 1998 was US$ 47 billion (FAO, 2000).

Similar to terrestrial production systems, husbandry conditions play an important role in successful production of aquacultural species. However, due to the extreme intimate interaction between fish and its aquatic environment, changes in this environment can, therefore, easily result in a disturbance of homeostasis within a fish (Wendelaar Bonga, 1997). The maintenance of internal homeostatic equilibria is essential for normal function of cells, animals and -indirectly- for the maintenance of populations. In case of disturbance, an animal will try to establish a new equilibrium. The animal's behavioural and physiological reaction to this disturbance is commonly called the stress response (Chrousos and Gold, 1992). Man-made (aquacultural) production systems can be regarded as environmental challenges or demands for the adaptive capacity of the animals involved. If animals are incapable to cope with these production environments, prolonged stress response might result in maladaptation. Energy-dependent activities like growth, maturation and disease resistance are affected under these circumstances.

The stress response in fish and its influence on other physiological processes have been studied extensively. For commercial purposes, stress is mainly considered as an indirect selection criterion for disease resistance, due to the tight relationship between stress and increased disease susceptibility (Snieszko, 1974). However, only few selection experiments with stress and/or disease resistance as main traits of interest have been initiated so far. These selection experiments are mainly carried out in commercial important fish species, like rainbow trout (*Oncorhynchus mykiss*, Pottinger and Carrick, 1999), Atlantic salmon (*Salmo salar*, Fevolden et al., 1991), gilthead seabream (*Sparus aurata*, Afonso et al., 1998) and common carp (*Cyprinus carpio*, Wiegertjes, 1995). So far, only one selection experiment aimed at creating strains with divergent antibody production has been carried out in the latter species (Wiegertjes, 1995).

In 1996, an NWO-ALW programme started, aimed to study the physiological strategies during acclimation to a temperature shock in common carp (See summary NWO-ALW programme for results). Next to the neuroendocrine regulation of the cold shock stress response and its effects on the immune and reproduction system, the genetic background of stress in common carp was studied with the ultimate aim to create isogenic lines with either a high or low stress response, to be used in future research.

However, like many important traits, the stress response of an organism is probably regulated by many genes, thus making stress a complex genetic trait to analyse. To investigate such a complex trait, reproduction techniques like gynogenesis and androgenesis are useful. These can be utilised to produce complete homozygous or heterozygous (= F_1 hybrid) isogenic strains in only two generations. In the first generation of homozygous double haploid (DH) individuals, the genetic variation (V_G) within a group of DH fish sharing the same sire or dam is equal to the additive genetic variation (V_A). Furthermore, the variation between progeny groups is also equal to V_A and this feature can be used to estimate genetic parameters like heritabilities (h^2), especially if the h^2 is expected to be below 0.35 (Bijma et al., 1997; Bongers et al., 1997). In the second generation of homozygous isogenic (obtained through andro- or gynogenesis) and heterozygous isogenic strains (obtained by crossing two homozygous individuals), the phenotypic variation (V_P) for a trait within such a strain is completely due to environmental sources (V_E), since genetic variation is absent. Furthermore, the variation between homozygous inbred strains is theoretically equal to $V_A + V_E$ and between F_1 hybrids to $V_A + V_D + V_E$, where V_D is the dominance variance. Comparing the performances of a number of such isogenic strains enables the estimation of phenotypic variation due to additive and non-additive genetic factors. Through these features, DH individuals and their progenies provide a powerful model for dissection of phenotypic variance.

The aim of the present thesis was to examine the genetic background of the stress response in common carp (*Cyprinus carpio* L.) using DH individuals obtained through androgenetic reproduction. As model stressor, a rapid temperature drop was used. Through selection of DH parents with extreme stress responses, isogenic strains with divergent stress responses were created. Both the DH parents and the isogenic strains were used to study the influence of additive and non-additive genetic and environmental effects on the stress response. Furthermore, a study was carried out to examine possible association of recently developed carp microsatellite markers with quantitative trait loci (QTL) influencing the stress response in carp.

In the next paragraph of this chapter, the two major pathways of the stress response in fish are described in more detail and the choice of stressor is explained. Paragraph 3 describes the processes of selection, (cross)breeding and use of genetic markers (in fish) and gives arguments for the choice of the breeding plan. Finally, paragraph 4 gives an overview of the work done within the framework of this thesis and describes the connection between the different chapters that form this thesis.

2 Stress in fish

In bony fish (teleosts), two major neuroendocrine pathways control the stress response. Although these pathways are comparable to pathways in mammals and other terrestrial animals, there are some distinct differences in the regulation and functions of the stress response in fish, mainly as a consequence of the aquatic environment.

2.1 Hypothalamus – sympathetic nerves – chromaffin cell axis

The first major neuroendocrine pathway in fish is the hypothalamus – sympathetic nerves – chromaffin cell (HSC) axis (Figure 1). This pathway results in the release of the catecholamines (CAs) epinephrine and nor-epinephrine in the general circulation under conditions that require enhanced oxygen transport and mobilisation of energy substrates. Therefore, the three main functions of CAs are (Randall and Perry, 1992; Wendelaar Bonga, 1997):

1) Increase the oxygen uptake through the gills by increasing the ventilation rate, the diffusional surface area in the gills, the permeability of the tight junctions between branchial epithelium cells and the blood flow through the gills.
2) Increase the blood oxygen transport capacity by increasing the haematocrit (increased numbers of circulating erythrocytes and swelling of erythrocytes) and increasing the affinity of haemoglobin for oxygen by blood plasma acidification and cytoplasmatic alkalinisation (Nikinmaa, 1992) .
3) Increase of plasma glucose by glycogenolysis of hepatic glycogen reserves.

In fish, the increase in diffusional surface and permeability of the tight junctions due to high circulating CA levels also stimulates passive water and ion fluxes in the gills. The passive influx of ions and efflux of water in seawater fish (hydromineral balance ↑) or the opposite fluxes in freshwater fish (hydromineral balance ↓) can, therefore, be seen as negative side effects of the stress-related CA release. Because the release of the CAs is mainly regulated through the sympathetic nerves, the reaction time between the onset

of the stress and the CA release is relatively short (< 3 min), but it depends on the severity and nature of the stressor and the fish species involved (Wendelaar Bonga, 1997).

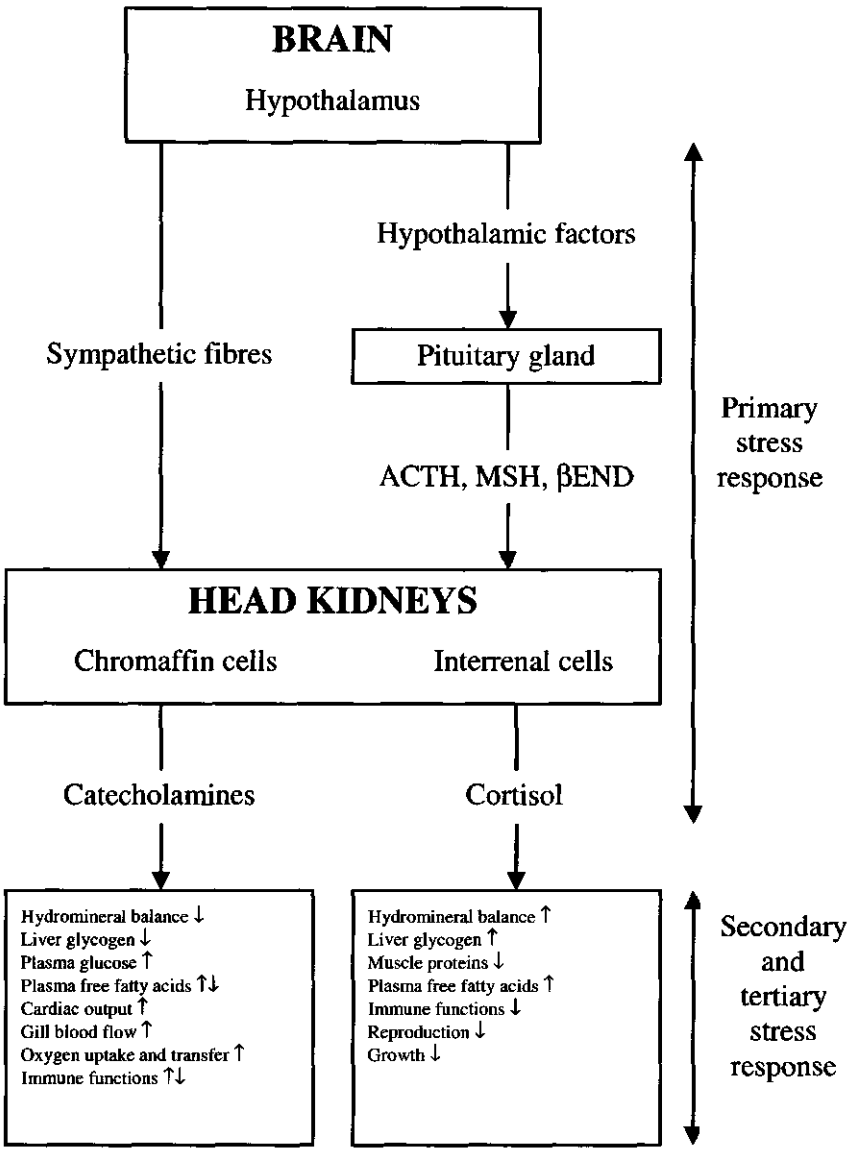

Figure 1: Generalised diagram of main neuroendrocrine elements of integrated stress response in teleost fish. ACTH: adrenocorticotropic hormone, MSH: Melanophore-stimulating hormone, βEND: β-endorphin, ↓ inhibitory, ↑ stimulatory (adapted from: Wendelaar Bonga, 1997).

2.2 Hypothalamus – pituitary – interrenal cell axis

The second major pathway controlling the stress response in fish is the hypothalamus – pituitary – interrenal cell (HPI) axis (Figure 1). In teleosts, this pathway mainly results in the release of cortisol as active steroid. The secretion of cortisol can be regulated by many different hormone substances including cortisol itself. However, the main regulatory hormones are adrenocorticotrophic hormone (ACTH) produced by the ACTH cells in the pituitary pars distalis and α-melanophore-stimulating hormone (α-MSH) potentiated by β-endorphin, which are both produced by the α-MSH cells in the pars intermedia of the pituitary gland (Pickering et al., 1986; Sumpter et al., 1986; Lamers et al., 1992). The release of ACTH is mainly regulated by the hypothalamic neuropeptide corticotrophin-releasing hormone (CRH) (Fryer, 1989; Olivereau and Olivereau, 1990, 1991). The α-MSH release is under control of a number of hypothalamic factors, including dopamine, thyrotropin-releasing hormone (TRH), melanophore-concentrating hormone (MCH) and CRH (Lamers, 1994; Lamers et al., 1994). Due to the neuroendocrine regulation of the cortisol release, increases of plasma cortisol occur somewhat slower than the increase in CAs, but increases can be observed already within 5 - 10 min after the start of the disturbance. The two major functions of cortisol in fish are related to the maintenance of the hydromineral balance (mineralcorticoid function) and the energy metabolism (glucocorticoid function). The mineralcorticoid function of cortisol is an additional function of cortisol compared to the functions of cortisol in terrestrial animals. This function of cortisol compensates the CA induced disturbances of the hydromineral balance, by stimulating the extrusion of Na^+ and Cl^- ions in seawater and the uptake of Na^+ and Cl^- ions in freshwater. However, a prolonged high plasma cortisol concentration can induce damage of skin- and possibly gill epithelium due to increased apoptosis (= programmed cell death) resulting in increased passive ion and water fluxes. The glucocorticoid action of cortisol is relatively slow compared to that of the CAs and the rapid rise in plasma glucose during acute stress is mainly caused by CAs. The main glucocorticoid effect is the redirection of energy destined for e.g. growth, immunity and reproduction towards behavioural and physiological processes involved in restoration of the disturbed homeostasis, thus enabling the animal to cope with the stressor (reviewed by Wendelaar Bonga, 1997).

For a detailed review of the stress response in teleosts see e.g. Pickering (1981), Barton (1997), Iwama et al. (1997); Wendelaar Bonga (1997), and Mommsen et al. (1999).

2.3 Measuring stress

Increased levels of circulating CAs and cortisol are most often used as indicator of a (primary) stress response (Figure 1). Especially cortisol is regarded as the stress hormone both in terrestrial and aquatic animals. However, both CA and cortisol levels change rapidly and are very sensitive to disturbances caused by e.g. sampling the animals. Therefore, in addition to the cortisol measurements, an indicator of the secondary stress response is often measured. These indicators include different metabolic (e.g. plasma glucose, lactate, liver and muscle glycogen), haematological (e.g. haematocrit, leucocrit, haemoglobin concentration), immunological (e.g. phagocytic activity, ratios of circulating B- and T-lymphocytes, antibody response to an antigen) and hydromineral (plasma chloride, sodium, osmolality) parameters (reviewed in Wedemeyer and McLeay, 1981; Adams, 1990; Barton and Iwama, 1991). In the present thesis, plasma cortisol was used as main indicator of the primary stress response. Technically, catecholamines can also be measured, but require rapid sampling since changes occur within minutes after onset of the stress and changes due to the sampling are confounded with those due to the actual stressor. As indicators of the secondary stress response, plasma glucose and lactate concentrations, and haematocrit and leucocrit percentages were used in some of the experiments. These parameters are also relatively easy to measure.

2.4 Temperature and stress

Due to the poikilothermic nature of fish, the environmental (water) temperature has a high impact on most physiological processes. Each fish species has a certain upper and lower temperature limit. Within these limits, a range is present in which physiological processes run optimally. The size of this optimal range and the heights of the upper and lower limits vary from species to species. An eurythermal species like the common carp can survive within the temperature range of 0 – 32°C (Aston and Brown, 1978), whereas a stenothermal species like the Antarctic eelpout (*Pachycara brachycephalum*) can survive within a temperature range of 0 – 9°C. (Van Dijk et al., 1999). Processes involved in the stress response are, therefore, also influenced by the water temperature. The rate at which cortisol levels are elevated due to stressors like netting and crowding seem to be positively related with the environmental temperature (Davis et al., 1984; Sumpter et al., 1985; Barton and Schreck, 1987; Davis and Parker, 1990). The effect of environmental temperature on the amount of cortisol released is less clear. Davis and Parker (1990) found higher cortisol levels in striped bass (*Morone*

saxatilis) netted at 25 – 30°C than in bass netted at temperatures ranging from 5 – 16°C. Similarly, Sumpter et al. (1985) found higher cortisol levels in brown trout (*S. trutta*) handled and confined at 13.4 than at 5°C. Conversely, Barton and Schreck (1987) did not find any influence of the environmental temperature on the height of the cortisol peak in chinook salmon (*O. tshawytscha*). Next to the rate and height of the cortisol response, the recovery after a stressor can also be influenced by temperature in different fashions. Davis and Parker (1990) found that in striped bass stressed at 25 – 30°C cortisol levels never returned to basal levels after the stress, but at temperatures between 10 and 21°C, cortisol levels returned to resting levels within 6 hours after the acute stress. On the other hand, Barton and Schreck (1987) found a slower recovery in chinook salmon kept at 7.5°C compared with fish kept at 12.5 and 21°C.

Besides the modulating role of the environmental temperature on the stress response due to other stressors, a sudden temperature change acts as a stressor by itself with a high physiological impact on fish. Gradual changes within the optimal range can be compensated before they produce noticeable disturbances of homeostasis. However, rapid temperature changes will lead to disturbances and, therefore, act as stressors. Both heat and cold shocks have been used to induce acute stress in different species of fish (Strange et al., 1977; Franklin et al., 1990; Sun et al., 1992, 1995; Le Morvan-Rocher et al., 1995), but the majority of the research on temperature effects focuses on (acute or chronic) heat stress. In the present study, we chose (three-hour) cold shocks as acute stressor.

2.5 The cold shock

Temperature shocks can be applied using a variety of techniques. For instance, Franklin et al. (1990) and Le Morvan-Rocher et al. (1995) applied their temperature shocks instantaneously by transferring the fish from one aquarium to another with a different water temperature. In such a design, however, the temperature stress is confounded with handling and confinement stress due to the netting of the fish. A possible temperature shock method without handling the fish is through heating or cooling the inlet water in an experimental unit. The rate of temperature change in such a design is highly dependent on the total water volume in the experimental units and the capacity of the heater or cooler. We used another approach allowing a rapid cold shock without handling. The experimental facilities used in the present thesis, consisted of three experimental units with two layers of eight rectangular 140 l aquaria each. All 48 aquaria were supplied by the same recirculation system and the total water volume in this recirculation system was *c.* 10 m^3. Using the available cooling device, cooling the

water from e.g. 25 to 16°C would still take considerable time (> 3 hours). Furthermore, the water temperature in all 48 aquaria in the three experimental units would decrease, which is both unnecessary and undesirable, since control (warm water) aquaria are also needed in an experimental design.

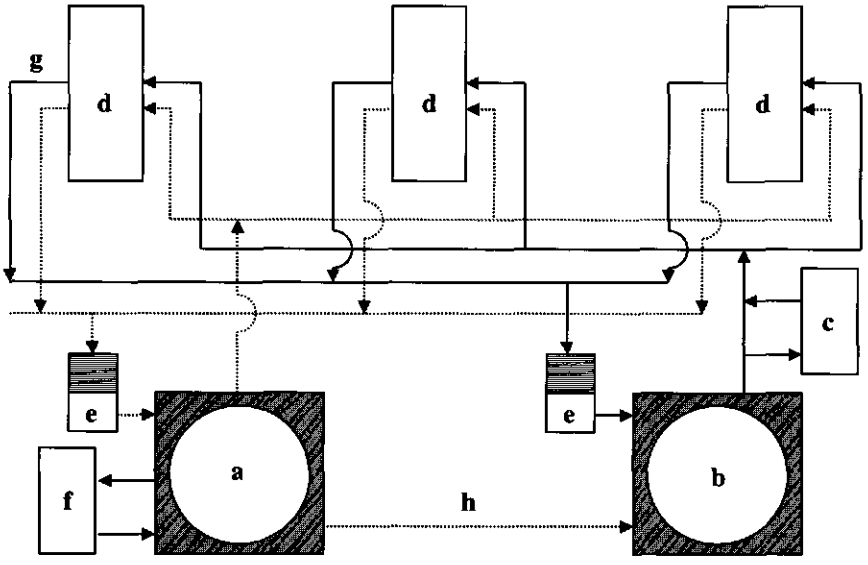

Figure 2: Schematic representation of the two recirculation systems used in the experiments and their respective water flows. (a) biological reactor cold water system (temperature adjustable 14 – 25°C), (b) biological reactor warm water system (25°C), (c) UV-filter, (d) experimental units consisting of two layers with eight 140 l aquaria each, (e) laminar flow sedimentation tank, (f) cooling machine. Between experiments, both systems were kept at 25°C and one warm water outlet (g) was connected with the 'cold water' outlet, thus creating a surplus of water in the 'cold water' system (a). This surplus water was returned to the warm water system (b) through a overflow (h).

To overcome these problems, all 48 aquaria were fitted with two inlet and (outlet) tubes connected to two separate recirculation systems (Figure 2). Under normal circumstances, the water temperature in both systems was kept at 25°C and both systems were connected (total water volume *c.* 16 m^3) resulting in similar water quality parameters (pH, dissolved oxygen, conductivity, NH_3-N, NO_2-N, NO_3-N). Two days prior to a cold shock, the systems were disconnected and the water in one system was cooled down to the desired temperature. On the sampling day, cold shocks could be initiated in any of the 48 aquaria by switching the inlet (and outlet) tubes from one

supply system to the other. Due to mixing, the desired lower temperature was reached *c.* 60 minutes after onset of the shock. This way, we were able to subject the fish to a rapid cold shock with selectable amplitudes without handling the fish.

3 Breeding in fish

The discipline of animal and plant breeding is a science developed in the last century. Today, the use of genetically improved farm animals and plants is common practice in agriculture. Conversely, selective breeding programmes are still rare in fish and shellfish and only a small percentage of the total aquaculture production is based on genetically improved stocks (Afonso et al., 1998; Gjedrem 1998). To secure the availability of aquatic products in the (near) future, the aquaculture production will need to increase by about 4.5% per year (Gjedrem, 1998). To help reach this goal, breeding programmes offer a valuable tool. There are two ways by which the breeder can change the genetic properties of a population. The first is by choosing the individuals to be used as parents (selection) and the second by control of the way the parents are mated (breeding). A short description of both approaches is given below (adapted from Falconer and Mackay, 1996). In the third paragraph, the use of different markers (phenotypic, allozyme, DNA) in these approaches is discussed.

3.1 Selection

The aim of selection is to increase the frequency of the desired alleles in the population. However, most traits of interest are controlled by a number of (major and minor) genes and there are environmental effects (= quantitative trait), which are completely hidden from us. Therefore, selection relies on measuring individual phenotypic values for the desired trait. The decision to select a certain animal as parent can be based solely on the performance of the animal itself, but also on performances of the relatives. Especially in fish breeding, where due to the higher fecundity of the fish larger full- and half-sib families can be produced, information from relatives can be more important than individual information. Furthermore, for some traits, selection of parents has to rely completely on information of siblings or progeny. For instance, selecting male fish that will produce progeny with good egg-quality can only be based on information of female relatives. However, the pedigree in a population has to be known to use this information.

The phenotypic value of an individual (P), measured as deviation from the population mean is the sum of two components. The deviation of the family mean from

the population mean (P_f) and the deviation of the individual from the family mean (P_w). The relative weight that is put on the components determines the type of selection.

Individual selection

If individuals are selected solely on the basis of their own performance (equal weight for P_f and P_w), the selection is called individual selection (or mass selection when the selected animals are mated *en masse*). This is a simple method and no pedigree information is necessary to select the individuals. However, for most commercial cultured fish species only a few parents are required to produce a new generation large enough to stock a farm. Therefore, individual selection can result in a rapid increase of the inbreeding coefficient (Gjedrem, 1998). Thus, to restrict the increase of inbreeding, the number of parents should be kept at a certain level. An optimum design for fish breeding programmes with constrained inbreeding when applying individual selection for a certain trait is discussed by Gjerde et al. (1996). Pottinger and Carrick (1999) used individual selection in rainbow trout to create lines with either a high or low stress-related cortisol response. In common carp, individual selection has been used, for example, to improve the growth rate (Moav and Wohlfarth, 1976), the body shape (Ankorion et al., 1992) and the resistance to dropsy (etiological agent: *Aeromonas* spp.; Kirpichnikov et al., 1993).

Family selection

If whole families are selected or rejected for further breeding purposes (weight on P_w is zero), the selection procedure is called family selection. The main circumstances under which family selection is preferred are: 1) the trait has a low heritability ($h^2 = V_A / V_P$; where V_P is the total phenotypic variance), and 2) there is little variation due to common environment (V_{E_c} is small), and 3) large families are available. Furthermore, family selection can be applied if the trait of interest cannot be measured on the individual itself, but can be measured on the family mean. The efficacy of this selection method rests on the fact that the environmental deviations of the individuals tend to cancel each other out in the mean value of the family. On the other hand, environmental variation common to all members in a family impairs the selection procedure. If this V_{E_c} is large, apparent genetic differences between families might actually be caused by differences in common environments. Furthermore, the larger the family the more accurate the mean phenotypic value will approach the mean genotypic value. A big disadvantage of family selection is the relative high inbreeding or conversely the low selection intensity that can be achieved. Therefore, a large number of families have to be selected for production of the next generation and, as such,

successful family selection with constrained inbreeding requires a lot of maintenance space and administrative work. An example of a breeding programme in fish applying family selection is discussed by Gjøen et al. (1997). The Atlantic salmon and rainbow trout F_1 lines with divergent cortisol responses used in the experiments described by Fevolden et al. (1991, 1992, 1993a,b) were made by family selection within the F_0 generation. In carp, the ongoing selection programme for dropsy-resistant Krasnodar carp strains has been conversed to family selection (Hulata, 1995).

Within-family selection

Two different selection schemes for within family selection can be distinguished, but the term 'within-family selection' is sometimes used to describe both, thus causing some confusion (Hill et al., 1996). The first is selection within-families (SWF), in which the best male(s) and female(s) are selected within a family on the basis of their own performance. The second is where individuals are selected across families based on their deviation from the family mean P_w (SDM; weight on P_f is zero). For SDM, it is unlikely that all families will contribute one male and one female to the next generation. The main condition under which these methods have an advantage over the others is a large V_{E_c} component influencing the trait. If families are kept in separate tanks, a large part of the variation between family means for e.g. growth might be caused by tank effects. Selection within families would eliminate this large non-genetic component from the variation operated on by selection. An important practical advantage of selection within families (SWF), especially in laboratory experiments, is that it economises breeding space (Falconer and Mackay, 1996). If family sizes are larger than four individuals, higher responses are predicted for SDM, because no constraints are made on selecting the best animals. The maximum relative difference is seen to be for families of about ten of each sex. If SDM is practised after correction for sex, using a common mean, its relative efficiency rises further. However, as the effective population size (N_e) is smaller for SDM than SWF, long-term responses are expected to be less (Hill et al., 1996).

Combined selection

Next to zero weights for either the P_f and P_w component or equal weights, other weights can also be put on the P_f and P_w components. This form of selection is called combined selection. The most accurate breeding value (A) estimate for an individual is h^2P. This can be applied to both components of P to obtain a formula for the best estimate of the expected breeding value:

$$E(A) = h_f^2 P_f + h_w^2 P_w \quad (1)$$

To maximise the use of the information of both P components, the best weights are, therefore, the heritability of family means (h_f^2) and the heritability of the within-family deviations (h_w^2). Using these heritabilities, the expected response of the three simple selection procedure and the combined one can be calculated and used to compare the efficiency of the different methods (see: Falconer and Mackay, 1996, Chapter 13). The relative merits of the different simple selection methods to that of combined selection are shown in Figure 3.

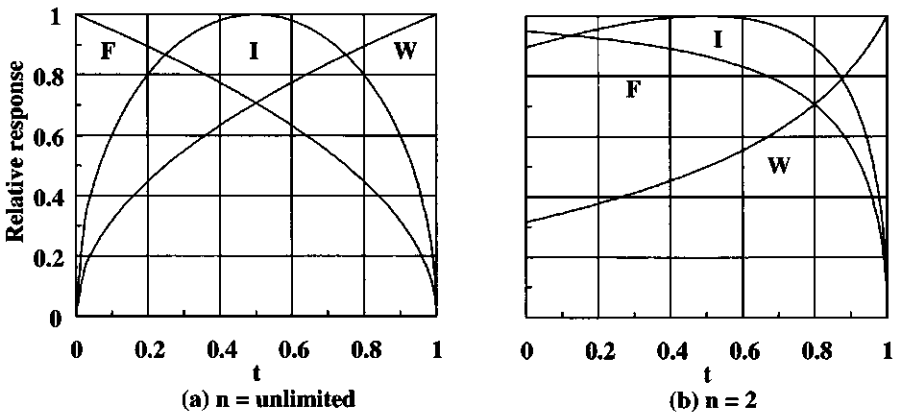

Figure 3: Relative merits of the different methods of selection, with full-sib families. Responses relative to that for combined selection plotted against the phenotypic intraclass correlation, t. t = resemblance of family members, I = individual selection, F = family selection, W = selection on deviation from family mean (SDM) and n = number of individuals per family. (Adapted from: Falconer and Mackay, 1996).

Comparing the three simple methods with combined selection shows that the three simple methods are never better than combined selection. On the other hand, however, the expected response of one or the other simple method is never more than 20% below that of the combined selection method. Comparing the three simple methods, individual selection is best over much of the range of t (= ½ (V_A + V_{E_c})/V_P = resemblance of family members). The reason for this is that individual selection operates on the whole of the additive genetic variance, whereas family selection operates only on the variance between family means, and within-family selection only

on the variance within families. A comparison between the efficiency of combined family selection and within family selection and individual selection only on growth rate in fish has been made by Gjøen (in Gjedrem, 1998). Under the same circumstances (population size: 3600; ΔF 1% / generation; h^2 = 0.20; mating ratio 1M:2F), individual selection yielded the highest relative genetic gain (100) compared with combined selection (93) and family selection (75).

Index selection

Next to the two sources of information (P_f and P_w) used in the four selection forms mentioned above, other sources of information might also be available. These sources include information from the parents, full-sibs, half-sibs and other relatives. This information can be combined to calculate an index on the basis of which the parents of the next generation are selected. Calculation and use of this index is, however, beyond the scope of this introduction. For a more detailed description see e.g. Nordskog (1978), Van Vleck (1993) and O'Flynn et al. (1992, 1999).

3.2 Inbreeding and crossbreeding

Inbreeding in itself is almost universally harmful and a breeder or experimenter normally seeks to avoid it as far as possible, unless it is desirable for some specific purpose. There are two main purposes to produce inbred lines. The first purpose of inbreeding is to use these inbred lines for subsequent crossing and exploitation of the hybrid vigour. Hybrid vigour, or heterosis, is the phenomenon that loss of performance due to inbreeding is restored when two inbred lines are crossed. In carp, heterosis for growth rate, survival or disease resistance have been reported in crosses of wild and domesticated European, Russian, Chinese and Japanese strains (reviewed by Hulata, 1995). However, evaluating the inbred strains and their crosses requires a lot of effort. The condition under which inbreeding followed by crossbreeding is a better means of improvement than selection is when much of the genetic variance is non-additive and/or most of the additive genetic variation has been exploited.

A second (more important?) purpose of inbreeding is the production of genetically uniform strains for research purposes. Through inbreeding, no genotypes are created that cannot occur in the base population, but the possibility of unlimited replication of a certain genotype (with a desired trait) is the main merit. Furthermore, individuals within a strain show less phenotypic variation. Through the use of genetically uniform strains, the replicability (variation between replicates in an experiment), the repeatability (variation between experiments in the same laboratory)

and the reproducibility (variation between experiments in different laboratories) can be improved (Festing 1979, Bongers et al., 1998). For this, both homozygous inbred and F_1 hybrid strains made by crossbreeding two homozygous parents are used in fish research.

As mentioned in paragraph 1, androgenesis is one of the reproduction techniques, which can be used to create isogenic lines. Androgenesis consists of two phases. In phase 1, the eggs, whose DNA has been destroyed by ultraviolet radiation, are fertilised by normal sperm. This produces a haploid zygote, in which all chromosomes come from the father. However, due to its haploid nature, it is not viable. To restore diploidy, a pressure or temperature shock is used to prevent nuclear division during the first cleavage of the zygote (phase 2). Due to the treatment and the fact that the offspring are 100% homozygous, the percentage of viable larvae is low (10-15%). In species like common carp, where the males are heterogametic (XY), 50% of the androgenetic offspring is female and 50% is supermale (YY). For a more detailed description of the androgenesis technique used in the present thesis see Bongers et al. (1994).

Previously, androgenesis was mainly used to produce 100% homozygous individuals (= doubled haploids, DH) and isogenic lines for research purposes. For commercial purposes, it was mainly used to produce viable supermales (YY), which when crossed with normal females would give 100% male progenies to be used in monosex culture. Next to these applications, DH individuals are increasingly used for linkage analyses, marker mapping and quantitative trait loci (QTL) association studies (e.g. Lie et al., 1994; Slettan et al., 1997; Young et al., 1998; chapter 5).

In an androgenetic DH progeny group, individuals are 100% homozygous but not identical due to Mendelian sampling. The additive genetic relations between a parent and its DH progeny are equal to 1.0 and the additive genetic relations within a DH progeny group are also 1.0. Therefore, the additive genetic variance within a DH progeny group is equal to the additive genetic variance between DH progeny groups, resulting in a doubling of the additive genetic variance (Bongers et al., 1997). Based on these principles, a DH progeny group can be used to estimate the breeding value of the parent and, consequently, DH progeny groups from different parents can be used to estimate heritabilities (Bijma et al., 1997; Bongers et al., 1997), an important genetic parameter for breeding purposes. In chapter 4, this design is used to estimate heritabilities for morphological and stress-related traits in common carp. Isogenic lines (homozygous or heterozygous) can also be used in breeding programmes. Because different traits can be measured on the same genotype, they can be used to estimate genetic correlations between traits. Furthermore, they are useful to estimate the presence

and magnitude of genotype × environment interactions and can function as internal control lines in long-term breeding programmes to estimate variances due to (changing) environmental factors (Van der Lende et al., 1998).

3.3 Genetic markers and their use in breeding.

At present, a huge variety of genetic markers is available through the use of molecular biological techniques. Previously, only morphological and biochemical markers were available. In common carp, scalation or pigment characteristics are often used as morphological markers. The big advantage of morphological markers is that the phenotypes can be scored on the spot and, in general, genotypes can be deduced directly or through pedigree information. However, the big shortcoming of morphological and biochemical markers is their relative low level of polymorphism. This problem can be solved by using DNA based molecular markers, which display a much larger level of polymorphism. Another (practical) advantage of DNA markers compared to biochemical markers is the relatively small tissue or blood sample needed for the analysis. Such samples can be taken without sacrificing the fish. Although biochemical markers also require laboratory equipment, the required equipment for most DNA markers has to be more sophisticated and requires specific expertise and skill, which is the biggest limitation for the use of DNA markers at this moment.

A wide array of DNA markers are available and can be grouped into clone/sequence based (CSB) and fingerprint (FP) markers (Dodgson et al., 1997). The first category requires the isolation of a cloned DNA fragment and often determination of some, if not all, of its DNA sequence. The CSB markers include among others microsatellites (or simple sequence repeat (SSR) markers; Tautz, 1989) and single nucleotide polymorphisms (SNP; e.g. Cooper et al., 1985; Landegren et al., 1998). The FP markers require no *a priori* knowledge of the sequence of the polymorphic region or isolation of a cloned DNA fragment, and include among others random amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams et al., 1990), minisatellites (Jeffreys et al., 1985) and amplified fragment length polymorphisms (AFLPs, Vos et al., 1995).

Microsatellite, minisatellite and satellite markers are based on repetitive DNA (variable number of tandem repeat (VNTR) markers; Nakamura et al., 1987) and the division between mini and micro mainly depends on the length of the repeating unit (Tautz, 1993), although the borderline (in base pairs, bp) between both can vary with different authors. Satellites are composed of repeat units of several thousand base pairs with repetition grades of 10^3 – 10^7 at each locus and are usually located in

heterochromatin, mainly in centromeres. Minisatellites have repetitive units of *c.* 9 – 65 bp and microsatellites consist of tracts of repeats of 1 – 9 bp. Both are distinctly different from satellites in that both mini- and microsatellites have only a moderate degree of repetition, the length of the repeat unit is shorter, and repetitive loci tend to be more dispersed throughout the genome (Debrauwere et al., 1997). In fish, microsatellites have been developed for an increasing number of species including most important salmonid species, Atlantic cod, sea bass, African catfish and European eel (Slettan et al., 1993; Brooker et al., 1994; Garcia de Leon et al., 1995; Galbusera et al., 1996; Daemen et al., 1997). It is estimated, that in the genome of fish, and vertebrates in general, over 10^4 – 10^5 microsatellites are present. However, in comparison with other vertebrates, microsatellite alleles tend to be longer in fish, resulting in a higher number of alleles and thus a higher level of polymorphism (Brooker et al., 1994).

The uses of genetic markers are numerous. In population genetic studies, markers are used e.g. to study the population structure within a species and the evolutionary relationship with other more or less related species (e.g. Jarne and Lagoda, 1996; Jorde et al., 1998). Similarly, markers are used in breeding programmes to identify progenies belonging to certain parent combinations (e.g. Galvin et al., 1995) or test the heterozygous or homozygous state of an individual (e.g. Corley-Smith et al., 1996). This latter application is very important in relation with androgenetic and mitotic gynogenetic reproduction, where produced offspring should be 100% homozygous in theory. However, residual heterozygosity has been reported in gynogenetic and androgenetic progenies (Thorgaard et al., 1985; Carter et al., 1991; Lin and Dabrowski, 1998). Next to their use in population genetics, genetic markers are used to create genome maps and study association of markers with single genes or QTLs influencing traits of interest. Genetic or linkage maps, essentially, provide flag-posts along the genome that allow locations to be identified (e.g. Hearne et al., 1992; Kocher et al., 1998; Young et al., 1998). They are obtained by analysing the co-segregation of alleles of different markers within pedigrees. Some genetic markers (e.g. microsatellites and SNPs) can also be physically mapped, providing information about the specific chromosomes the markers are located on and their orientation. The genetic maps are mainly used to enable the mapping of genes affecting (commercially) important traits (e.g. Jackson et al., 1998; Sakamoto et al., 1999). These genes may be identified as single genes inherited in a Mendelian fashion (e.g. the scalation genes in carp), or they may be regions of the genome identified as accounting for a significant proportion of the variation in a trait that is quantitative in nature (QTLs). Once association of markers with important genes has been established, marker information can be used in selection

programmes for traits regulated by these genes (marker assisted selection, MAS). In such cases, segregation information of associated linked marker alleles provides information about the (co-)segregating gene alleles. This MAS is especially useful for traits that are difficult and expensive to measure or traits which can only be measured after the selection has been carried out. However, MAS requires a comprehensive performance recording and typing of the animals involved in the selection procedure.

For common carp, 32 microsatellites had already been developed prior to the start of the present programme (Crooijmans et al., 1997), but during the work described in the present thesis, 48 additional carp microsatellites were developed. Currently, no genetic map of common carp has been published.

4 Selective breeding for stress in common carp using androgenesis (outline of the thesis)

Our aim was to produce homozygous and heterozygous isogenic strains of common carp with divergent stress responses due to a cold shock to be used in future research. As a preparatory step, a number of experiments were carried out to investigate the validity of the cold shock as a stressor and to define the selection criterion. The amplitude needed to induce a noticeable stress response in carp and plasma cortisol, glucose and lactate dynamics during and after such a cold shock were studied (**Chapter 2**). Based on these results, the plasma cortisol concentration at 20 min after onset of a single 9°C cold shock was set as selection criterion in our selection experiment. Furthermore, a first impression of the influence of non-genetic effects on the stress response was obtained by studying the effects of environment, age, and repeated cold shocks on the response during and after a cold shock.

The first step in the actual selection experiment (Figure 4) was the formation of the base population (**Chapter 3**). This base population was an F_1 cross between six sires from a wild strain originating from the Anna Paulowna (AP) polder and a highly domesticated homozygous E4 dam already present in our laboratory. The wild origin of the AP carps was verified through genetic characterisation of the F_1 fish using biochemical and genetic analyses i.e. allozymes and carp microsatellites. As a first step towards the production of isogenic strains, the F_2 generation was made by androgenetic reproduction of 33 randomly picked sires from the six E4×AP full-sib families. These 33 progeny groups were subjected to a cold shock, thus enabling us to estimate heritabilities for different stress-related parameters (**Chapter 4**) and select the highest and lowest responding families for further selection (**Chapter 6**). The heritability

($h^2 = V_A/V_P$) of a trait is an important parameter used to determine the form of the selection programme and to predict its success. Although h^2 estimates for stress-related cortisol were available in Atlantic salmon (h^2 = 0.07) and rainbow trout (h^2 = 0.27) (Fevolden et al. 1993b) proving the existence of a genetic basis for stress in these species, no estimate for h^2 for stress-related parameters was available for common carp. Because the model used to estimate the h^2 assumed a complete homozygous state of the animals and to ensure that only homozygous individuals would be used for subsequent production of the homozygous and heterozygous isogenic strains, all 660 individuals were characterised with 11 microsatellites (**Chapter 5**). In addition, the microsatellite data and morphological and stress-related data obtained in chapter 4 were used to examine the segregation of the microsatellites, the linkage between microsatellites and possible association of microsatellites with the phenotypic traits. The actual selection procedure and the rationale for subsequent family and individual selection is described in **Chapter 6.** The selected sires and dams were used to create homozygous inbred and heterozygous isogenic strains. These strains were used to study the influence of additive genetic, non-additive genetic and environmental effects on the stress response. In chapter 2, the 'complete' stress response pattern was tested in only one isogenic strain, thereby limiting the observations to a single genotype. Simultaneously with the selection programme, two experiments were carried out to test the 'complete' response pattern in four other isogenic strains. As in chapter 2, the possible influence of age on the response pattern was examined. The results from these experiments are described in **Chapter 7**. In **Chapter 8** the overall results and possible implications of these results are discussed.

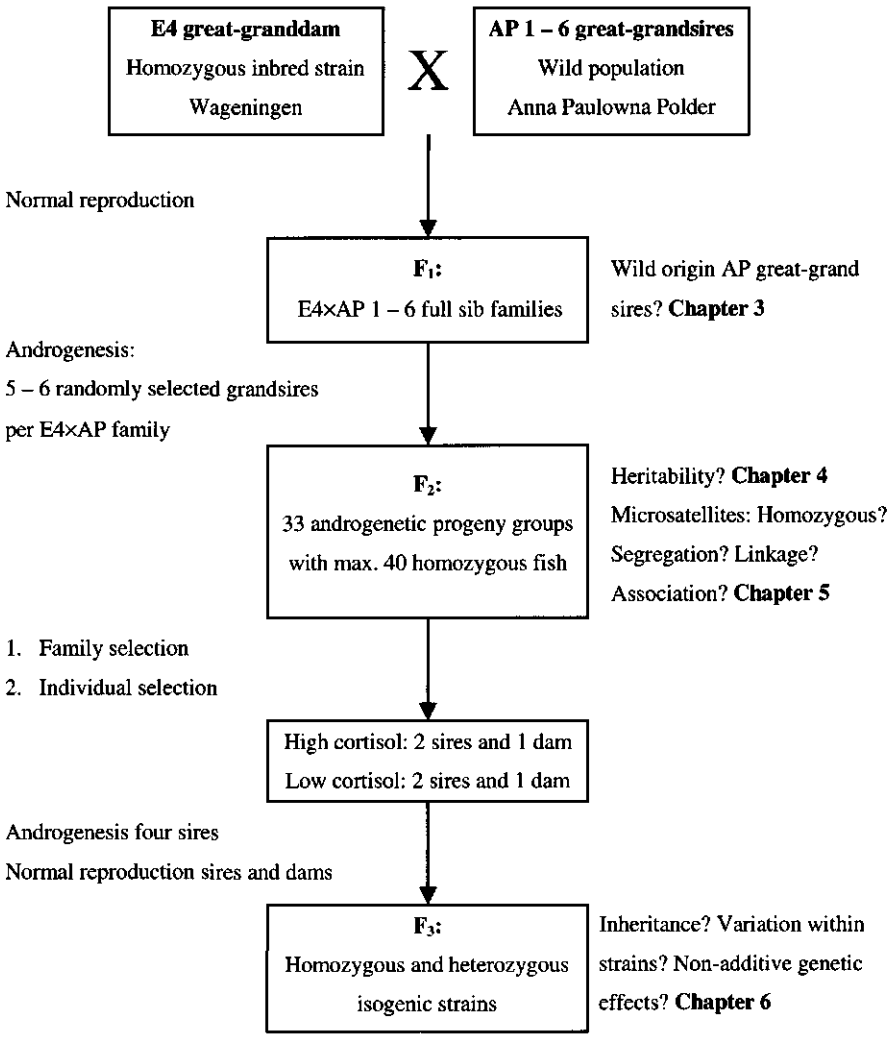

Figure 4: Schematic representation of the selection experiment aimed at producing isogenic strains of common carp with divergent stress response due to a cold shock. On the left-hand side, the reproduction and/or selection method applied to obtain the next generation is given. On the right-hand side, the most important question(s) per generation is (are) given together with a reference to the specific chapter.

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Chapter 2

Cold shocks: a stressor for common carp

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Abstract

The stress response of common carp (*Cyprinus carpio* L.) was studied by evaluating plasma cortisol, glucose and lactate after single or multiple rapid temperature drops (ΔT: 7, 9 or 11°C). All three amplitudes used induced a significant rise in plasma cortisol levels. Peaks occurred within 20 min after onset of the cold shock. No stress-related secondary metabolic changes were observed in any of the experiments described: plasma glucose levels remained unaffected and plasma lactate levels dropped. Carp of 60 days old showed a significant stress response, although plasma cortisol levels were lower than those observed in carp of 120 days. Furthermore, fish that had experienced multiple cold shocks showed an overall lower cortisol response than fish experiencing a single cold shock, indicating that habituation to this stressor occurred.

1 Introduction

For fish, changes in environmental conditions may result in disturbance of homeostasis. The animal tries to minimise or eliminate the deleterious effects of such changes by behavioural and physiological adjustments, which are called collectively the stress response (Pickering, 1981; Chrousos and Gold, 1992). Rapid temperature changes, either heat or cold shocks, are among the stressors with a high physiological impact on fish (Crawshaw, 1979). Within the lethal temperature limits of an organism, a tolerance range of temperatures, to which a species can acclimatise, can be discerned as well as a narrow optimal temperature range in which the efficiency of most or all physiological processes is maximal, with optimal conditions for growth, reproduction and disease tolerance. In general, the temperature tolerance ranges are narrower for poikilothermic aquatic animals, than for their terrestrial counterparts (Blaxter, 1992). Temperature changes of the water have immediate effects on fish because of the high rate of heat exchange between the animal and the ambient water. Such changes require highly complex adjustments of virtually all body processes, because many enzymes have different Q-10 values and, therefore, most multi-enzyme processes are disturbed during a temperature change. Gradual changes in temperature, within the tolerance zone, can be coped with before they produce noticeable disturbances of internal equilibria. However, rapid changes will disturb internal homeostasis and, thus, by definition act as stressors.

Sudden temperature changes, both of natural origin (e.g. daily variations in water temperature in shallow waters or thermocline in deeper water bodies) or caused by human activities (e.g. thermal discharges), represent a very common threat to fish,

the effects often being additive or synergistic with those of other adverse stimuli (e.g. low water pH, algae, oxygen shortage) (Wagner et al., 1997). Study of such temperature changes has relevance for fish in natural waters as well as in aquaculture conditions (Crawshaw, 1979; Pickering, 1981; Barton and Schreck, 1987; Donaldson, 1990). However, although some effects of temperature and (gradual) temperature changes on the stress response have been investigated (Sun et al., 1992, 1995; Ryan, 1995; Wagner et al., 1997), little is known about the effects of rapid temperature drops on the stress response of fish.

In this paper, the effects of rapid temperature decreases are examined in the common carp *Cyprinus carpio* L. This species was chosen, because of its relative broad temperature tolerance range (optimal temperature range: 15 – 32°C; Coutant, 1977), thus enabling us to use large temperature decreases without reaching the tolerance limits of the species under investigation. The experiments aim to assess whether and to what extent these rapid temperature drops induce a stress response in common carp, and whether carp can habituate to such conditions.

2 Materials and methods

2.1 Fish

In all experiments described, an isogenic strain of common carp was used (E4×R3R8). This strain is used commonly in the Netherlands for endocrinological, immunological and toxicological studies (Wiegertjes et al., 1994; Gimeno et al., 1996; Arends et al., 1998; Bongers et al., 1998). Four different batches of fish were raised using the same standard rearing procedure. Larvae were divided into groups of *c.* 300 larvae and housed in 25 l aquaria at 25°C in a recirculation system consisting of a plate separator, a biological reactor and a UV-treatment unit. The number of groups depended on the amount of fish required for the different experiments. During the first 21 days post-hatching (dph), the larvae were fed to satiation three times a day with freshly hatched *Artemia* nauplii. After this period, the surviving fry (> 95%) were mixed and randomly divided into groups of 120 animals. These groups were moved to the experimental unit with two rows of eight 140 l aquaria, filled with 100 l of water. Fish were fed a commercial trout pellet (Provimi, 91 series, Rotterdam, The Netherlands) at a daily ration of 20 g $kg^{-0.8}$ until 120 dph. At 60 dph, each group was split further into two groups of 60 fish each. From 120 dph onwards, the fish were fed a fixed ration of 1.5% of body weight twice a day (0930 and 1500 hours), based on the weight at 120 dph.

Thereafter the fish were no longer weighed. The mean weight and age of the fish in the different experiments are shown in Table 1.

Table 1: Mean weight (g), age (days post hatching), number of shocked (n_s) and control (n_c) aquaria, number of fish per aquarium (n_f), amplitude (°C) of the cold shock used and number of shocks (n_{sk}) during the different experiments. Experiments 1 to 3 were started at 1000 hours, experiment 4 started at 1200 hours and experiments 5a and 5b started at 0930 hours.

Experiment	Weight mean (SD) g	Age dph	n_s	n_c	n_f	Amplitude	n_{sk}
1	121.1 (28.1)	177	8	8	10	7°C	1
2	104.3 (17.3)	163	8	8	10	9°C	1
3	114.8 (23.4)	137	8	8	8	11°C	1
4	158.5 (20.1)	169	8	8	10	9°C	1
5a	4.9 (1.0)[a]	60	3	2	10	11°C	26
5b	62.7 (19.9)	120	3	2	10	11°C	1

[a] Weight at first shock

2.2 Cold shock

Rapid temperature changes can be applied by transferring fish from one tank to another, but this might impose additional handling stress, which could complicate our proper understanding of the temperature stress. Therefore, a system was created in which the aquaria of the experimental unit received water from two parallel water supply systems connected to separate recirculation units. Before an experiment, both systems were inter-connected and the water was kept at the standard rearing temperature of 25°C. Two days prior to the experiment, the water supply systems were disconnected and the water in one system was cooled down to 18, 16 or 14°C. By doing so, the water quality parameters of the two supply systems were similar. On the day of the experiment, a cold shock was initiated by switching the 25°C inlet tube of the aquarium to the inlet tube of the cold water system. The flow of both supply systems was set at 5 l min^{-1}. The desired shock temperature was reached after 60 min. The outlets of the aquaria receiving cold water were placed in a separate gully belonging to the cold water

recirculation system, thus preventing mixture of water of the different supply systems. Three hours after onset of the shock, the procedure was reversed and the water temperature returned to 25°C in 60 min. As a consequence of this set-up, the three temperature drops (7, 9 and 11°C) resulted in different rates of temperature change, $\Delta T.\ t^{-1}$ (°C min^{-1}).

2.3 Experiments

In total, five experiments were carried out to investigate the stress response of carp after a single or after multiple cold shock(s).

Experiments 1 – 3 examined the effect of different cold shock amplitudes on the stress response during and after the shock. These three experiments started at 1000 hours. One month prior to each experiment, 160 fish were divided randomly over the 16 aquaria within an experimental unit. Eight randomly chosen aquaria were subjected simultaneously to a cold shock. At t = 0, 20, 40, 60, 90, 120, 180 and 300 min after onset of the shock the fish of one shocked aquaria and of one control aquaria were sampled. The amplitude of the cold shock used was 7, 9 or 11°C (Table 1). Fish were not fed on the day of the experiment.

Results (unpublished) from an experiment, carried out in the same laboratory to investigate a possible circadian rhythm in carp, showed some variation in plasma cortisol concentrations during the day (range 10 – 50 ng ml^{-1}), with two distinct peaks (> 100 ng ml^{-1}) at 1000 and 1500 hours. Although the fish were not fed at the sampling day as in the present experiments, these peaks did coincide with the approximate times they were normally fed. Experiment 4 started at 1200 hours instead of 1000 hours to investigate the possible role of the first feeding peak (1000 hours) on the plasma cortisol levels measured in experiments 1 – 3. The sampling schedule was identical to that of experiments 1 – 3.

In experiment 5, the combined effects of age and habituation to cold shocks were investigated. Eight aquaria situated in one row were stocked with 20 fish each. At 60 dph three randomly chosen aquaria were subjected to an 11°C cold shock. Fish in the other five aquaria did not receive a cold shock. At 0, 20 and 60 min after onset of the shock, one shocked and one control (non-shocked) aquarium were sampled. After anaesthetising all twenty fish, ten were chosen randomly for blood sampling and the remaining ten were put back in the original aquarium. Between 60 and 120 dph, the fish in the three aquaria, which were shocked at 60 dph, were subjected to multiple 11°C cold shocks at a rate of three per week (habituation effect). The days and starting time

of these cold shocks were chosen randomly. At 120 dph, these fish experienced their 26th cold shock. Fish from the three additional aquaria, used as controls at day 60, also received a cold shock on day 120 (age effect). Both multiple- and single shocked groups were sampled at 0, 20 and 60 min after onset of the shock. The fish in the two remaining aquaria were also anaesthetised at day 60 and ten randomly selected fish were put back in the aquarium. At day 120, they served as controls at 20 and 60 min after start of the shock. On sampling days, the fish were fed half an hour prior to the onset of the cold shock.

2.4 Blood sampling and plasma analyses

All fish in an aquarium were caught by one sweep of a tightly fitting net and anaesthetised immediately in water containing 0.3 g l^{-1} tricaine methane sulphonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) and 0.4 g l^{-1} $NaHCO_3$ (pH *c.* 7.5). Blood samples were taken by puncture of the caudal vessels using 1 ml syringes fitted with a 23 G needle and rinsed with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany) as anti-coagulant. Blood was transferred to Eppendorf vials and centrifuged immediately for 10 min at 1500× ***g*** and 4°C. Plasma was stored at -20°C pending analyses. To determine haematocrit and leucocrit values, freshly collected blood was spun down in a haematocrit centrifuge (11330× ***g***).

Plasma samples were analysed for cortisol, glucose and lactate concentrations. Cortisol levels were determined by radioimmunoassay (RIA) (De Man et al., 1980; Van Dijk et al., 1993). Plasma glucose was analysed with a commercial kit (no. 124 028, Boehringer Mannheim GmbH, Mannheim, Germany) using a 96 well microtitre plate. The final solution was measured at 420 nm. Lactate concentrations were analysed using a lactate kit (no. 735-10, Sigma Aldrich, St. Louis, MO, USA) adapted to be used with microtiter plates (measurement wavelength: 540 nm).

2.5 Calculations and statistical analyses

The experimental set-up chosen in the different experiments does not preclude possible aquarium effects. Due to technical limitation, it was not possible to include replicates of each sample point tested (both control and shock). Instead, fish were considered as the experimental unit. The variation between the control points reflects the background variation due to tank effects, disturbances during sampling, etc.

All statistical analyses were carried out using the SAS software package (SAS Institute Inc., 1990). Data for weight, haematocrit, leucocrit, cortisol, glucose and lactate were tested per experiment for significant differences by ANOVA using the parametric General Linear Model procedure. For each parameter, the residues of the models were normally distributed (Wilk-Shapiro test, $W > 0.90$) and showed equal variances (Levene's test), thus making transformation of the data unnecessary. Differences in cortisol, glucose and lactate concentrations per time point were analysed using the Student's two-tailed *t*-test. Correlations between different parameters were expressed as Pearson correlation coefficients (*r*). *P*-values < 0.05 were regarded significant.

3 Results

Differences in temperature profiles among aquaria proved to be negligible (Figure 1).

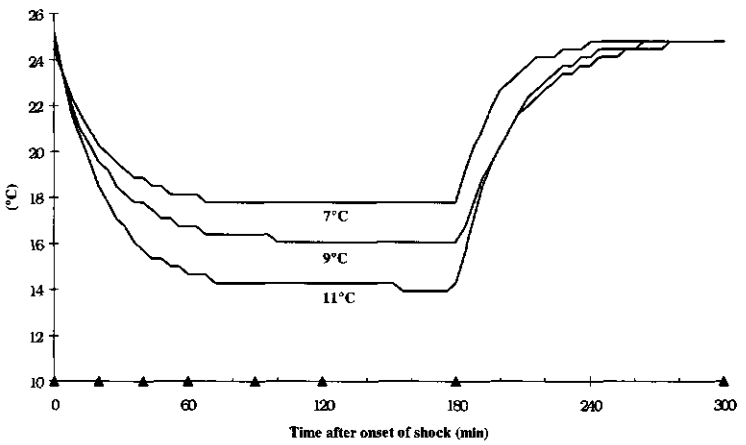

Figure 1: Temperature profiles of a 3 h cold shock with 7, 9 and 11°C amplitude. Triangles represent the sample points during experiments 1 – 4.

Plasma cortisol levels in the controls ranged from 5 – 85 ng ml^{-1} (Figure 2). In experiments 1 – 3, cortisol concentrations in the controls were highest at the beginning of the experiments. In experiment 4, these higher initial levels did not occur. All three cold shock amplitudes used (Figure 2) induced a significant elevation in plasma cortisol. Peak levels (Mean ± SD) of 193 ± 52, 205 ± 59 and 265 ± 37 ng ml^{-1} were found after

20 min in experiment 1 (7°C, Figure 2a), 2 (9°C, Figure 2b) and 4 (9°C, Figure 2d), respectively. In experiment 3 (11°C, Figure 2c), the highest concentrations were found at 20 and 40 min namely 330 ± 59 and 338 ± 43 ng ml^{-1}, respectively. In general, plasma cortisol levels in the shocked groups were significantly elevated above those in the matching control groups throughout the cold shock.

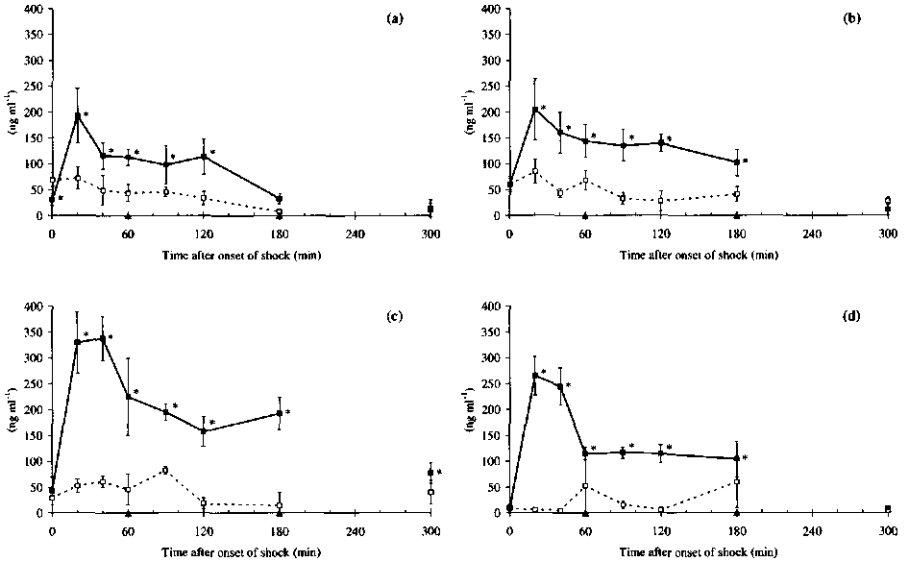

Figure 2: Plasma cortisol concentrations (mean ± SD; ng ml^{-1}) during a 7°C (a), 9°C (b) and 11°C (c) cold shock started at 1000 hours and a 9°C (d) cold shock started at 1200 hours. Significant ($P < 0.05$) differences between the control (-□ -) and shock (-■-) groups are indicated by asterisks. Triangles mark the point at which the lowest water temperature is reached (60 min) and the point at which the temperature starts rising again (180 min).

In experiment 1, the concentrations found at 180 min in both groups were not different. Two hours after the cold shock (t = 300 min), when temperatures were back at 25°C (Figure 1), cortisol levels in the shocked groups were similar to those of the control groups, except in experiment 3. Although the plasma cortisol concentration had decreased, compared to the level found at the end of the cold shock (t = 180 min), it was significantly higher than the concentration found in the matching control group. A correlation between sampling order of the fish within a group and plasma cortisol was not found in any of the experiments.

No significant differences were observed in glucose levels between control and shocked fish at any given sample point except in experiment 2, where glucose levels in the control groups were significantly higher than those in the corresponding shock groups. Mean (± SD) plasma glucose concentrations were 2.40 ± 0.32, 4.75 ± 1.11, 2.36 ± 0.42 and 1.79 ± 0.30 mmol l^{-1} in experiments 1 to 4. In experiments 2, 3 and 4, significant positive correlations (r = 0.479, 0.284 and 0.169, respectively) were found between the sample order of the fish (within a group) and plasma glucose.

Significant differences for lactate values between control and shocked groups were found starting 40 – 60 min after onset of the shock (Figure 3a – d). A significant decrease in mean lactate concentrations compared with the controls was detected in the cold shocked groups during a 9 or 11°C cold shock. Two hours after the cold shock, lactate levels had returned to control levels again. For lactate a significant positive correlation (r = 0.235) between sampling order and plasma lactate was found in experiment 2

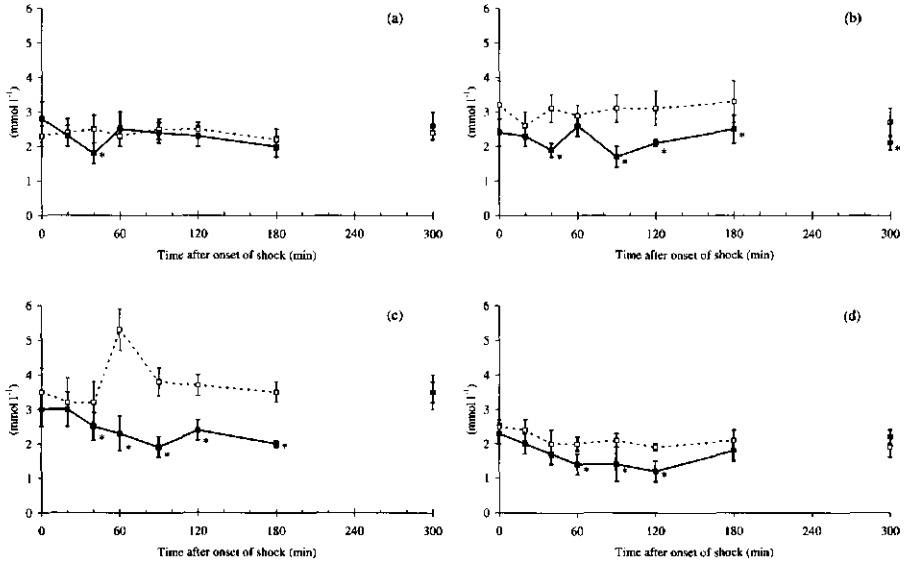

Figure 3: Plasma lactate concentrations (Mean ± SD; mmol l^{-1}) during a 7°C (a), 9°C (b) and 11°C (c) cold shock started at 1000 hours and a 9°C (d) cold shock started at 1200 hours. Significant ($P < 0.05$) differences between the control (-□ -) and shock (-■-) groups at a sample point are indicated by asterisks.

Cold shocks used in experiment 1 and 2 did not influence haematocrit or leucocrit values. Mean values (±SD) were 42.0 ± 4.3 and 1.2 ± 0.5% for haematocrit and leucocrit, respectively in experiment 1, and 44.3 ± 4.1 and 1.6 ± 0.5% in experiment 2. A significant positive correlation between sample order and haematocrit was found in both experiments (r = 0.337 and 0.474 for experiment 1 and 2, respectively).

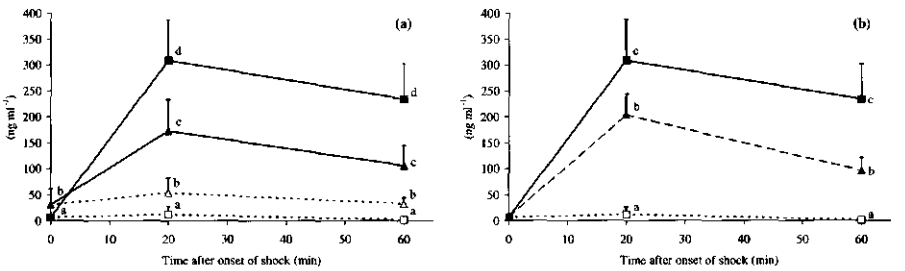

Figure 4: a) Plasma cortisol concentrations (Mean ± SD; ng ml^{-1}) during a single 11°C cold shock in a control (- Δ -) and shock (-▲-) group at 60 days post hatching (dph) and a control (-□ -) and shock (-■-) group at 120 dph. Letters denote significant ($P < 0.05$) differences between groups at a sample point. b) Plasma cortisol concentrations (Mean ± SD; ng ml^{-1}) in a group receiving a single 11°C cold shock (-■-), a group receiving their 26th 11°C cold shock (-▲ -) and a control group (-□ -) at 120 dph. Letters denote significant ($P < 0.05$) differences between groups at a sample point.

Cortisol levels in carp subjected to a single 11°C cold shock at 60 and 120 dph (age effect) were significantly higher than those found in the matching controls at 20 and 60 min after onset of the cold shock (Figure 4). Peak levels (mean ± SD) of 173 ± 60 and 309 ± 79 ng ml^{-1} were recorded at 20 min at 60 and 120 dph, respectively. Control levels at 60 dph were significantly higher than those observed at 120 dph at all three sample points. Cortisol levels at 20 and 60 min in the shocked groups sampled at 60 dph were significantly lower than those shocked at 120 dph.

Because fish were fed in experiment 5 prior to the cold shocks, glucose dynamics are different compared with experiments 1 – 4 (Table 2). A single shock at 60 dph led to a significantly reduced increase in glucose compared to the increase of plasma glucose with the non-shocked controls. At 120 dph, the glucose levels in control and single shocked groups remained stable. The multiple shocked group had a significantly lower glucose level at 60 min after onset of the shock compared with the

control group. For plasma lactate, a significant decrease was observed in all shocked groups on both sample days. There was no significant difference between the single and multiple shocked groups at 120 dph.

Table 2: Mean plasma glucose and lactate concentrations (SD between brackets) (mmol l^{-1}) at 60 and 120 days post hatching of different groups receiving no, a single, or multiple cold shocks. Samples were taken at 0, 20 and 60 min after onset of the 11°C cold shock. Letters denote significant ($P < 0.05$) differences per sample point.

	Glucose (mmol l^{-1})			Lactate (mmol l^{-1})		
	Control	Shock	M. Shock	Control	Shock	M. Shock
60						
0'	2.90 (2.30)	-	-	3.43 (0.73)	-	-
20'	5.51 ^{a} (1.07)	3.78 ^{b} (1.07)	-	3.43 ^{a} (1.16)	1.79 ^{b} (0.68)	-
60'	9.37 ^{a} (2.57)	4.59 ^{b} (1.25)	-	4.31 ^{a} (1.38)	1.79 ^{b} (0.48)	-
120						
0'	11.93 (1.06)	-	m*	2.88 (0.39)	-	m*
20'	12.19 (1.99)	12.22 (1.31)	11.20 (1.50)	2.95 (0.31)	2.62 (0.50)	2.68 (0.26)
60'	12.60 ^{a} (1.46)	11.01 ^{ab} (1.34)	10.18 ^{b} (1.19)	3.12 ^{a} (0.42)	1.47 ^{b} (0.14)	1.52 ^{b} (0.19)

* Missing value

4 Discussion

4.1 Plasma cortisol dynamics

Although catching induces stress in fish, noticeable stress responses, as reflected by e.g. high plasma cortisol levels, can be avoided if blood samples are taken within 10 – 12 min after netting combined with anaesthesia (Franklin et al., 1990; Weyts et al., 1997). The present sampling was organised in such a way that < 10 min elapsed between netting and blood sampling. Furthermore, tricaine methane sulphonate (TMS) was used as anaesthetic, based on the experience that plasma cortisol concentrations remain relatively stable after TMS anaesthesia (Iwama et al., 1988). As a result mean

basal plasma cortisol levels in controls were in general < 30 ng ml^{-1} and are comparable with levels found in other experiments with carp (Van Dijk et al., 1993; Yin et al., 1995; Pottinger, 1998). Looking at the basal levels, it can be stated that the experimental procedure successfully prevented a noticeable cortisol stress response related with catching and sampling of fish and that the cortisol responses measured in the shocked groups were due entirely to the cold shock.

However, control levels found during the first hour of experiments 1, 2 and 3 were somewhat elevated (> 50 ng ml^{-1}). The shorter intervals between the first sample points and the disturbance caused by sampling cannot be the reason, since the same sampling procedure was used also in experiment 4 (Figure 2d) and in this experiment the initial elevation of the control levels was absent. The results in experiment 4 indicate that the higher initial levels observed in experiments 1 – 3 were probably caused by the cortisol peak, occurring around the time they are normally fed (unpublished results). Similar results were reported by Spieler and Noeske (1984), who found that goldfish *Carassius auratus* L. fed at fixed times for a prolonged period (unaffected by the time of the day feeding occurred) showed a rise in plasma cortisol prior to the feeding time. Starting an experiment two hours after normal feeding time (Experiment 4, Figure 2d) or feeding the fish prior to an experiment (Experiment 5, Figure 4a,b) seems to avoid these higher initial cortisol levels.

All three amplitudes used in the different experiments induced a significant rise in plasma cortisol levels within 20 min after onset of the shock, indicating that the cold shocks used were experienced as stressors by the fish. The fact that peak levels were already found within 20 min after onset of the shock instead of after 60 min, when the final temperatures were reached, indicates that the change in water temperature might cause the response and is the real stressor, rather than the temperature amplitude *per se*. The temperatures experienced by the fish are within the optimal temperature range of common carp (15 – 32°C; Coutant, 1977) and thus the fish may be anticipated to cope with such temperatures. The cortisol levels found in cold shocked groups at 20 min after onset of the shock are in the same range as those found in carp by Yin et al. (1995), Weyts et al. (1997) and Pottinger (1998), who used either crowding, netting or rod-and-line capture as stressors. However, peak cortisol levels were considerably lower than levels (> 750 ng ml^{-1}) reported by Van Raaij et al. (1996) using deep hypoxia as stressor, indicating that cold shocks impose a relatively mild stress.

Although the cortisol dynamics observed during the different shocks are more or less similar, there are some striking differences especially at the last sample points.

Comparison of the cortisol concentrations at the last sample point during the shock (t = 180 min) shows, that the level in the 7°C cold shocked group is no longer significantly higher than the level found in the matching control group, whereas in the experiments using 9 or 11°C cold shocks levels in the shocked groups are still significantly elevated, indicating a prolonged response, a suppressed cortisol turnover or a lower overall metabolism. Furthermore, at the last sample point (t = 300 min), when temperature is back at 25°C, cortisol concentrations were back to control levels in all three experiments with either a 7 or 9°C amplitude, but not in the experiment with the 11°C amplitude. Although no samples were taken during the temperature increase of the experiments, again it seems that the time required for recovery is related directly to the amplitude of the cold shock. Nevertheless the typical peak response together with the relatively quick recovery (< 4 h) are in accordance with results found by other authors (Pickering et al., 1982 (handling); Einarsdottir and Nilssen, 1996 (water level reduction); Pottinger, 1998 (rod-and-line capture)) and indicate that the cold shocks applied were not experienced as long-lasting stressors.

4.2 Plasma glucose and lactate dynamics

A rapid stress-related hyperglycaemia, as often observed in fish, including common carp (Van Raaij et al., 1996; Pottinger, 1998), could not be detected in any of our experiments. This rapid rise in plasma glucose concentration is generally observed when either hypoxia or increased activity of the fish are (part of) the stressor used (Pickering, 1981; Van Raaij et al., 1996; Pottinger, 1998). It can be argued, that neither hypoxia nor increased activity occurred during the cold shock, which might be a reason for the absence of a rapid hyperglycaemia. However, more extensive research, including determination of catecholamine levels during the cold shock, is necessary to clarify the reason for the absence of glucose elevation in the present experiment.

Sun et al. (1992, 1995) observed a significant hyperglycaemia in tilapia *Oreochromis niloticus* L. subjected to a slower 14 – 16°C temperature drop, but this hyperglycaemia became noticeable only after at least 24 h after the start of the experiment. This hyperglycaemia was most likely cortisol-related, since cortisol is a more slow-acting glucocorticoid (Van der Boon et al., 1991). Because in experiments 1 – 4, the last sample was taken only 5 hours after the start of the experiments a cortisol-related plasma glucose increase might have occurred after the sampling was stopped.

An (oxygen) stress-related increase in lactate levels as reported by Dabrowska et al. (1991) and Van Raaij et al. (1996) in carp did not occur in these experiments also.

However, cold shocks with a 9 or 11°C amplitude caused a decrease in plasma lactate levels, which was similar to the findings in tilapia subjected to a temperature decrease (Sun et al., 1992, 1995). This decrease is probably temperature- and not stress-related. Possibly, alterations in enzyme activities, similar to the observed activation of lactate dehydrogenase by low temperatures in rainbow trout *Oncorhynchus mykiss* Walbaum (Somero and Hochachka, 1969), might play a role in the plasma lactate level decrease observed in the present experiment.

During certain phases of the TMS anaesthesia hypoxia occurs, which causes a number of physiological changes, comparable with secondary stress responses, including increased concentrations of blood glucose, lactate and haematocrit (Brown, 1993). Indeed positive correlations between these parameters and the sampling order were found in the present experiments, but, because of the short sampling time, changes within these parameters remained within the range of basal levels published by other authors (Dabrowska et al., 1991; Van Dijk et al., 1993; Yin et al., 1995). However, the significantly higher plasma glucose concentrations found in experiment 2 may be ascribed to the longer residence of the fish in TMS before sampling and to the time elapsed between sampling of the first and the last fish (which was > 12 min in this experiment). Furthermore, sampling procedures, used in this particular experiment, resulted in a longer sampling time for the control groups, explaining why glucose concentrations found in the control groups in experiment 2 are higher than those found in the shocked groups, whereas in the other experiments no significant effect could be found. On the other hand it enhanced the magnitude of the decrease found for lactate (Figure 3b). This sampling problem was avoided in experiment 4 and, therefore, these lactate values are more likely to reflect lactate dynamics during a 9°C cold shock.

4.3 Age effect and habituation

There are nearly no reports of age or size affecting a stress response in fish. Stouthart et al. (1998) showed that carp larvae (0 – 5 dph) already have a functional pituitary-interrenal axis immediately after hatching, as concluded from a significant cortisol response after handling. However, the developmental stage of fish could influence the (absolute) amount of cortisol released during a stress response (Pottinger, 1998). The results from experiment 5 show that 60-day-old carp show a significant cortisol response, but concentrations found at 20 and 60 min are lower than those found in fish subjected to a similar cold shock at 120 dph. Pottinger (1998) found differences in stress response, which were possibly related to size or age of the carp used. The stress

response in larger carp (± 500 g) was lower than the response in smaller carp (45 – 120 g). However, both the 60 and 120 dph carp in experiment 5 are more or less within the weight range of the smaller group and a possible age-related attenuation of stress responsiveness might occur at a later age.

Next to age, sexual maturity might also play a role in the stress response observed, since the all-male carp became mature between 60 and 120 dph. However, increased plasma testosterone (T) or 11-ketotestosterone (11-KT) levels would probably result in a lower stress-related cortisol response similar to the results found by Pottinger et al. (1996), who found a decrease in cortisol response during confinement in immature rainbow trout *O. mykiss* implanted with either T or 11-KT containing cocoa butter pellets.

The lower cortisol levels found in the multiple shocked group at 20 and 60 min compared with the single shocked group at 120 dph, indicate that habituation to cold shocks might occur. A similar finding was reported by Einarsdottir and Nilssen (1996), who demonstrated that Atlantic salmon *Salmo salar* L., subjected every third day to an acute stressor (water level reduction), showed a 42% reduced maximum level and a faster recovery after the fifth challenge compared with the maximum level and recovery time found after the first challenge. However, given the experimental set-up used in the present experiment, a role of other factors like exhaustion of the interrenal cells or an altered clearance rate or feed-back mechanism can not be excluded.

This study shows that a rapid decrease in water temperature induced a consistent cortisol stress response in carp, with no apparent secondary effects. Furthermore, carp appear to habituate to these temperature shocks. However, it is not known whether the observed response pattern is typical for carp in general or whether it is strain specific. Experiments in the same laboratory indicate that, while the cortisol response pattern to a 9°C cold shock is consistently the same for the strain used in these experiments, different responses were found when other genotypes were used (chapter 4). This suggests the existence of genetic variation for responses to temperature changes in common carp.

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Chapter 3

Genetic characterisation of wild Dutch common carp (*Cyprinus carpio* L.)

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Abstract

Six male carp, caught in the water system surrounding the Anna Paulowna (AP) Polder in The Netherlands, were characterised using allozyme and microsatellite markers. At the sMDH-A1,2* loci an allele was found, which was previously only found in wild River Rhine and wild Vietnamese common carp. Microsatellite allele frequencies showed, that these AP carp were significantly different from a group of carps originating from several different domesticated strains. Based on both allozyme and microsatellite data, the AP carp most likely originated from a wild or feral self-sustaining population.

1 Introduction

Common carp (*Cyprinus carpio* L.) has a long history of domestication both in Europe and Asia (Balon, 1995). Although the existence of several self-sustaining wild or feral populations has been documented (Paaver and Tammert, 1993; Kohlmann and Kersten, 1999), truly wild populations of common carp are probably rare (Komen, 1990). In The Netherlands, in co-operation with the Organisation for Improvement of Inland Fisheries (OVB), we were able to capture a number of carp in the water system surrounding the Anna Paulowna (AP) Polder. Since no carp had been released into this system for more than 30 years (OVB, personal communication), these carp most likely originated from a self-sustaining wild or feral population.

In the present paper, F_1 progenies from these AP carp were genotyped using both allozymes and microsatellites. The allozyme alleles found in these progenies were compared with alleles reported in wild and domesticated strains by Kohlmann and Kersten (1999). Microsatellite alleles found in the AP group were compared to those found in a group of domesticated carp (DOM group). The latter consisted of six individuals from six domesticated strains. These strains originally came from different regions within Europe and Israel, and as such, this DOM group covers a broad area within the geographic distribution range of European common carp. Results from both genotyping methods will enable us to assess the possible wild origin of the AP carp.

2 Materials and methods

2.1 Fish

The Anna Paulowna (AP) group consisted of six mature male carp (AP1 – 6), which were caught in the water system surrounding the AP Polder (Province of Noord Holland, The Netherlands). Since these fish could not be sacrificed for allozyme analysis, an F_1 progeny was made. However, making a complete AP progeny was impossible, as all female AP carp spontaneously ovulated in the tanks shortly after capture. Therefore, fresh milt samples were collected from the six AP sires and used to produce six full-sib families (E4×AP1 – 6) by crossing each AP sire with a homozygous E4 dam.

The domesticated (DOM) group consisted of six individuals originating from six different domesticated strains: Dor-70 from Dor, Israel (Moav and Wohlfarth, 1976), R3 from Chybie, Poland and R8 from Szarvas, Hungary (both described by Irnazarow, 1995), and W and the homozygous inbred strains E4 and E5 from Wageningen, The Netherlands (Komen et al., 1991). The number of fish per strain that were originally transported to Wageningen and their background can be found in Komen (1990)

2.2 Allozyme typing

All E4*x*AP full-sib families were reared separately until fish were large enough for tissue sampling. In total, 25 randomly selected E4×AP1 – 6 fish and three E4 fish were transported to the Department of Inland Fisheries (FVB.IGB) in Berlin, where they were sacrificed for tissue sampling (muscle and liver). Both strains were examined for their genetic variability in four enzymatic systems representing six gene loci (Table 1). Methods used for this analysis are similar to those described in Kohlmann and Kersten (1999) and allele frequencies found were compared with those found in wild and domesticated carp populations described in their publication. The nomenclature for designation of the loci and alleles follows the recommendations of Shaklee et al. (1990).

Table 1: Names, E.C. number, abbreviation, number of loci, tissue, alleles and allele frequencies of enzymes screened in the two strains. The relative electrophoretic mobilities of the alleles are given between brackets.

Enzyme	E.C. number	Abbreviation	Number of loci	Tissue	Alleles	Allele frequencies	
						E4	E4*x*AP
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3PDH*	1	Muscle	*a* (*100*)	1.0	1.0
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-B*	2	Muscle	*a* (*-100*)	1.0	1.0
Malate dehydrogenase	1.1.1.37	*sMDH-A*	2	Liver	*a* (*100*)	1.0	0.86
					b (*150*)	0	0.09
					c (*120*)	*0*	0.03
					d (*60*)	0	0.02
Phosphoglucomutase	5.4.2.2	*PGM*	1	Muscle/Liver	*a* (*100*)	1.0	1.0

2.3 Microsatellite typing

Per full-sib family, 10 randomly picked individuals were blood sampled by puncturing the caudal vessels using 1 ml syringes fitted with a 23 G needle rinsed with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany) as anti-coagulant. Blood was transferred to 2 ml tubes and stored at –80°C pending analyses. Per full-sib family, the individual blood samples were pooled and genomic DNA was isolated using the Puregene DNA isolation kit (Gentra systems Inc., NC, USA).

Together with DNA samples from the six domesticated individuals (Dor-70, R3, R8, W, E4 and E5), the six E4×AP DNA samples were analysed with 28 carp microsatellite markers (Microsatellite Fish Wageningen, MFW; Crooijmans et al., 1997). PCR was carried out as described by Crooijmans et al. (1996) and ten multiplex sets of PCR amplification products were made based on expected allele lengths (Table 2). The mixtures were separated on a 6% denaturing polyacrylamide gel (Sequagel-6, National Diagnostics, Atlanta, GA, USA) using an ABI 373 automated sequencer (Perkin Elmer, Foster City, CA, USA). Interpretation of the gels was done using Genescan and Genotyper software packages (Perkin Elmer).

The E4 and E4×AP1 – 6 microsatellite allele patterns were used to reconstruct the genotypes of the six AP sires (AP group). The genotypes of the six AP and six DOM individuals were used to 1) calculate the percentage of polymorph loci per individual and group, and number of alleles/locus, 2) examine differences between the DOM group and the AP group using Fisher's R×C test (Raymond and Rousset, 1995) and Fisher's combined probability test, and 3) estimate Nei's (1978) unbiased genetic distance between groups and pairs of individuals and construct a dendrogramme using UPGMA and bootstrapping. All analyses were carried out using the TFPGA software package (Miller, 1997). In the text, values for different parameters are expressed as mean ± SD.

Table 2: Multiplexing sets used to characterise the different wild and domesticated common carp with 28 Microsatellites Fish Wageningen (MFW). Per fluorescent dye (FITC, HEX or TET) the order of the MFWs reflects the ascending expected lengths of the amplification products.

	Microsatellite Fish Wageningen (MFW) no:		
Set no:	FITC	HEX	TET
1	6, 20		
2	18, 24, 21		
3	17, 25		
4	26, 29, 30		
5	1, 31		
6	9, 2		
7	15, 28	38	
8	14, 7, 5	39	
9	10		34, 35, 33
10	19, 22	42	

3 Results

The alleles found at the four enzymatic systems from E4 and E4×AP are shown in Table 1. The E4 strain was homozygous at all loci. The E4×AP strain was only heterozygous at the sMDH-A1,2* loci, at which four different alleles were found. Based on allozyme data only, Nei's (1978) unbiased genetic distance between E4 and AP was 0.02.

Based on the microsatellite analysis, the percentage of polymorph microsatellite loci for the domesticated Dor-70, R3, R8, W, E4 and E5 individuals were 57, 36, 43, 46, 0 and 0%, respectively. The mean percentage of polymorphic loci in the DOM group was 46% ± 9 (excluding E4 and E5). Percentages of polymorphic loci for the six AP sires were 46, 68, 54, 43, 43 and 61%, respectively (Mean AP group: 53% ± 10). The mean percentages for the DOM and AP group were not significantly different (*t*-test, $P > 0.05$). The mean numbers of alleles per locus were 3.0 ± 0.9 and 3.0 ± 1.3 for the

DOM and AP group, respectively (Overall mean: 3.9 ± 1.4 alleles/locus). For 13 loci (46%), the AP group had some 'unique' alleles that were not present in the DOM group. Vice versa, the DOM group had some 'unique' alleles for 17 loci (61%). Within these loci, both the AP and DOM group showed some 'unique' alleles at nine loci.

Fisher's combined probability test showed, that the AP group was significantly different from the DOM group (χ^2: 175.2, 52 df, $P < 0.0001$). Fisher's R×C test resulted in significant differences between the groups at 14 of the 28 loci. Nei's (1978) unbiased genetic distance between the AP and DOM group was 0.25. Nei's (1978) distances between pairs of individuals are shown in Table 3. The largest genetic distance (D = 0.88) was found between the R8 and E4 individuals. Between a number of individuals the genetic distance was zero (Table 3, Figure 1). Results from the bootstrap test showed, that the AP individuals clustered in 77% of 1000 replicates. The W, E4 and E5 individuals clustered in all replicates. The occurrence of the other clusters ranged from 24 to 53% of the replicates.

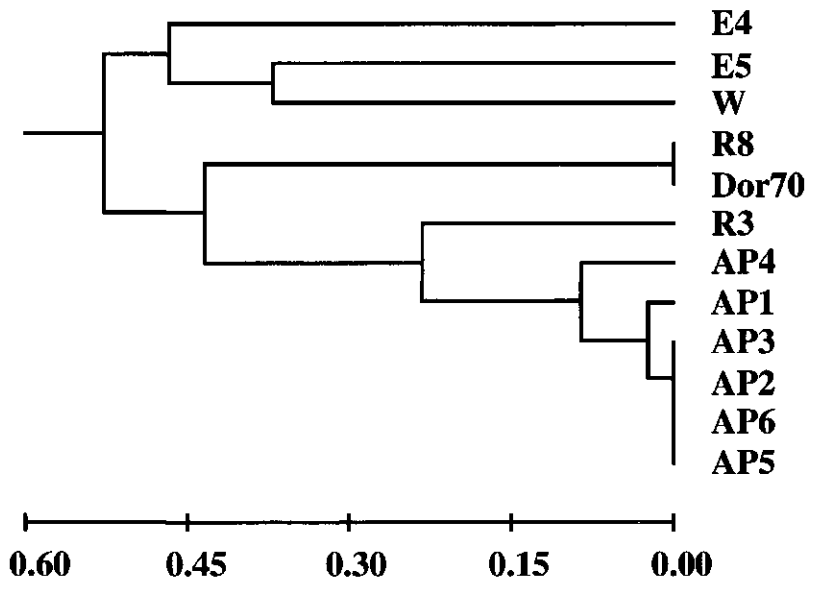

Figure 1: UPGMA Dendrogramme of Nei's (1978) unbiased genetic distance for 12 individual common carp of wild or domesticated origin. AP1 – 6 are individual carp caught in the Anna Paulowna Polder in The Netherlands; Dor70, W, R3, R8, E4 and E5 represent one individual of these domesticated carp strains.

Table 3: Matrix of unbiased genetic distance (Nei, 1978) between pairs of individuals based on microsatellite allele frequencies. AP1 – 6 are individual carp caught in the Anna Paulowna Polder in The Netherlands; Dor70, W, R3, R8, E4 and E5 represent one individual of these domesticated carp strains.

	Dor70	**W**	**R3**	**R8**	**E4**	**E5**	**AP1**	**AP2**	**AP3**	**AP4**	**AP5**	**AP6**
Dor70	xxx											
W	0.41	xxx										
R3	0.30	0.39	xxx									
R8	0.00	0.45	0.32	xxx								
E4	0.71	0.55	0.45	0.88	xxx							
E5	0.61	0.37	0.49	0.58	0.39	xxx						
AP1	0.58	0.63	0.30	0.57	0.54	0.54	xxx					
AP2	0.33	0.38	0.06	0.31	0.37	0.41	0.00	xxx				
AP3	0.42	0.31	0.23	0.39	0.60	0.75	0.00	0.00	xxx			
AP4	0.52	0.46	0.35	0.54	0.53	0.57	0.07	0.00	0.05	xxx		
AP5	0.46	0.63	0.29	0.44	0.61	0.61	0.07	0.00	0.03	0.23	xxx	
AP6	0.39	0.45	0.17	0.51	0.34	0.56	0.03	0.00	0.00	0.14	0.00	xxx

4 Discussion

As expected, E4 was homozygous at all six allozyme loci, and both E4 and E5 were homozygous at all 28 microsatellite loci investigated. Of the four enzymatic systems, E4×AP fish were only heterozygous at the sMDH-A1,2* loci. The estimated genetic distance between E4 and AP based on allozyme loci was much smaller than the one estimated using the microsatellite data. Partly this was caused by the difference in number of loci investigated (6 vs. 28), but also because microsatellites are in general more polymorph than allozyme loci, resulting in larger distances (Tessier et al., 1995; Estoup et al., 1998). Nevertheless, important information was obtained through allozyme analysis in the present study, since comparison of the allele frequencies between E4 and E4×AP showed that the *b, *c and *d alleles originated from the AP sires only. Kohlmann and Kersten (1999) found sMDH-A1,2 *a, *b and *c alleles in both domesticated and wild carp populations. However, the *d allele was only found in wild River Rhine and wild Vietnamese carp but in none of the investigated domesticated populations.

Based on Fisher's combined probability test and the dendrogramme, it can be stated, that the AP individuals as a group are significantly different from the DOM individuals presently available at our facilities. A conclusion illustrated by the fact that notwithstanding the diverse (geographic) origin of the individuals in the DOM group, the AP group had 'unique' alleles at 46% of the loci examined. Although the mean observed heterozygosity was somewhat higher in the AP fish compared to the DOM fish (excluding homozygous strains E4 and E5), this difference was not significant.

Based on all genotypic data obtained in the present study, it can be stated that AP carp most likely originated from a wild or feral population. A statement supported by some phenotypic characteristics of the AP fish, like an elongated (torpedo-shaped) and fully scaled body shape. Similar to the River Rhine population (described by Lelek, 1987 and Kohlmann and Kersten, 1999), the question remains whether the AP carp are pure (European) wild carp or a hybrid between wild and domesticated carp. However, the AP fish will constitute an important enrichment of the genetic variability within the broodstock presently available at Wageningen University. As such, they will be included in (selective) breeding programmes and future experiments to study, among others, the effects of domestication.

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Chapter 4

Heredity of stress-related cortisol response in androgenetic common carp (*Cyprinus carpio* L.)

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Aquaculture, accepted

Abstract

The aim of this paper was to estimate the heritability for the intensity of the stress-related cortisol response in common carp (*Cyprinus carpio* L.) using androgenetic progeny groups. For this, 660 androgenetic individuals (age 110 days) were subjected to a 9°C cold shock and blood sampled 20 minutes after onset of the shock. Heritabilities were estimated for weight, length, condition factor (K), and plasma cortisol, glucose and lactate concentrations using Gibbs sampling. Estimated heritabilities for the morphological traits weight and length were 0.09 (90% Highest Posterior Density range: 0.03 – 0.17) and 0.11 (0.04 – 0.21), respectively. The condition factor (K), showed a medium heritability of 0.37 (0.20 – 0.62). Heritabilities for basal plasma glucose and lactate were 0.19 (0.10 – 0.33) and 0.56 (0.33 – 0.85), respectively. For stress-related cortisol increase a high heritability estimate of 0.60 (0.37 – 0.90) was found. Although the height of this cortisol heritability has to be regarded with some reservation, due to confounding of some environmental effects with sire effects, the estimated heritability clearly shows that the intensity of the stress response due to a cold shock is hereditary in the carp population used.

1 Introduction

In aquaculture, selective breeding programmes are used progressively to increase e.g. growth rate, food conversion efficiency, meat quality or disease resistance (Gjedrem, 1983; Hulata, 1995). Stress resistance is occasionally included in such selective breeding programmes, as it is recognised to be a predisposing factor for reduced performance and increased disease susceptibility (Snieszko, 1974; Fevolden et al., 1993a). Previous research has shown, that selection for a high or low stress response to confinement is effective in fish species like Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Fevolden et al., 1993b; Pottinger and Carrick, 1999; Fevolden et al., 1999). Common carp (*Cyprinus carpio* L.) is a major species in aquaculture and used extensively as laboratory animal (Bongers et al., 1998). Strains with divergent stress responses would be very valuable for both fundamental and applied research and commercial carp culture. However, at present, no evidence for heredity of the intensity of the stress response in common carp has been reported.

In teleosts, cortisol is the main glucocorticoid released during stress (Chester Jones et al., 1980) and plasma cortisol concentrations can be used as a robust index of stress (Barton, 1997). As a standard stressor, confinement is often used (e.g. Pottinger and Carrick, 1999; Fevolden et al., 1999). However, confinement is a complex stressor involving handling, crowding and (if not properly executed) adverse environmental

conditions and, therefore, fish subjected to this stressor are prone to show increased environmental variation. In our laboratory, we use cold shocks as stressor with peak plasma cortisol levels at 20 minutes after shock onset as the main parameter (see chapter 2). While technically more difficult to realise, this stressor is highly reproducible, which is an important experimental advantage, where variation due to random environmental circumstances should be minimised.

Only a few reports are available in which androgenetic or gynogenetic designs are utilised to estimate variance components (e.g. Del Valle et al., 1996; Bongers et al., 1997b; Umino et al., 1997). Androgenetic progenies, technically known as doubled haploids, have the advantage over conventional progenies in that the additive genetic variance is doubled (Bongers et al., 1997b), thereby increasing the accuracy of the heritability estimations. The same design also allows for the production of high and low responding homozygous inbred strains in only two generations. An additional advantage of androgenesis over gynogenesis in carp is the presence of both male (yy) and female individuals in the progeny groups, which makes subsequent production of (isogenic) F_1 hybrid strains much easier.

Therefore, the aim of the present paper was to obtain heritability estimates for stress-related cortisol in common carp using androgenetic progeny groups with a broad genetic basis

2 Materials and methods

2.1 Sires

Although truly wild populations of common carp are rare, both in Europe and Asia (Komen, 1990; Kohlmann and Kersten, 1999), we were able to capture some carps in a semi-isolated water system (Anna Paulowna (AP) Polder). Genetic characterisation of these carp indicated that they originated from a self-sustaining wild population (see chapter 3). An F_1 consisting of six full-sib families (E4×AP1-6) was produced by crossing six wild AP males with one highly domesticated, homozygous, E4 female. Per full-sib family, five to six sires were randomly picked for androgenetic reproduction.

2.2 Androgenesis

Due to practical limitations, only 6 androgenetic progeny groups could be produced on a single day. In total, eight sessions were needed to obtain the desired number (>30) of progeny groups. In all sessions, isogenic E4E5 females (Komen et al., 1991) were used as egg donor. Eggs and sperm were obtained as previously described by Komen et al. (1991). Androgenesis was performed using the method described by Bongers et al. (1994). Briefly, for each male, approximately 1000 eggs were irradiated with a total UV-dose of 175 mJ cm^{-2}. Thereafter, the eggs were fertilised with milt and placed in the incubation-system (24°C). Thirty minutes after fertilisation, a two minute heat shock was administered by transferring the eggs from the incubation-system to a water bath of 40°C.

The mean yield (± SD) of diploid androgenetic larvae in the different sessions was 13.9 ± 2.3%. In the controls for UV-irradiation (UV irradiated eggs fertilised with sperm, but not heat shocked), the mean percentage of normal diploid larvae was 0.8 ± 0.9%.

2.3 Rearing of progeny groups

The first 21 days post hatching (dph), each progeny group was reared separately in 25 l aquaria and fed with freshly hatched *Artemia* nauplii to satiation three times a day. Mortalities were registered on a daily basis and progeny groups with less than 80 surviving larvae after 4 dph were culled. During this period, the mean survival percentage (± SD) in the androgenetic progeny groups was 47.4 ± 22.5%. At 21 dph, all 33 progeny groups were moved to 140 l aquaria connected to a 25°C recirculation system consisting of a plate separator, a biological reactor and an UV treatment unit. Fish were fed a commercial trout pellet (Provimi, 91 series, Rotterdam, The Netherlands) at a ration of 20 g $kg^{-0.8}$ d^{-1}. The amount of feed was adjusted daily assuming a feed conversion ratio of 1 and the fish were weighed every 14 days. At 85 dph, grossly deformed fish were culled and the number of fish per progeny group reduced to 40 randomly chosen individuals. From this day onwards, the fish were fed a ration of 15 g $kg^{-0.8}$ d^{-1}. Fish were not fed on the day of the cold shock. In the period between 21 and 110 dph, the mean survival percentage was 84.2 ± 10.6%.

2.4 Experimental design

A total of 33 androgenetic progeny groups were subjected to a standardised 9°C cold shock at an age of 110 dph. Because of the different hatching dates, shocking and sampling of the different androgenetic progeny groups was divided over eight days. The androgenetic progeny groups were housed in three experimental units consisting of two rows of eight adjacent aquaria each. All aquaria in the experimental units received water from the same recirculation system. Although possible individual tank effects could not be precluded, the experimental design enabled us to estimate possible effects of the experimental unit, the tank position within a unit (upper vs. lower row, right vs. left side) and the sampling day on the phenotypic traits recorded.

2.5 Cold shock

The cold shock was performed as described in chapter 2. In brief, all aquaria within the experimental units received water (25°C) from two interconnected water purification systems. Two days prior to a cold shock day, the two systems were disconnected and the water in one system was cooled down to 16°C. By doing so, the water quality parameters of the two systems were similar before and during a cold shock. The cold shock was initiated by switching the inlet and outlet tubes of the aquarium from the 25°C to the 16°C system. The desired shock temperature was reached after 60 minutes (flow rate 5 $l\,min^{-1}$). After three hours, the procedure was reversed and the water temperature returned to 25°C in one hour (Figure 1).

2.6 Sampling procedure

All fish (max. 40) in an aquarium were caught in one sweep with a tightly fitting net and immediately anaesthetised in water containing 0.3 $g\,l^{-1}$ Tricaine Methane Sulphonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) and 0.4 $g\,l^{-1}$ $NaHCO_3$ (pH approximately 7.5). After two minutes, when the fish were adequately anaesthetised, all fish were simultaneously removed from the anaesthetic and 20 fish were randomly picked to be sampled. All 20 fish were sampled within 10 min after capture. Blood samples were taken by puncturing the caudal vessels using 1 ml syringes fitted with a 23 G needle rinsed with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany) as anti-coagulant. The blood was transferred to 2 ml tubes and immediately centrifuged for 10 minutes at 1500× ***g***. Plasma and blood cell pellets were stored separately at -20°C pending analyses. Before the fish were put back into a recovery

tank, individual body weight and standard length were scored. Subsequently, all fish were moved back to the original aquarium. The applied sampling procedure prevents the occurrence of a noticeable cortisol stress response related with catching and sampling as of the fish (see chapter 2).

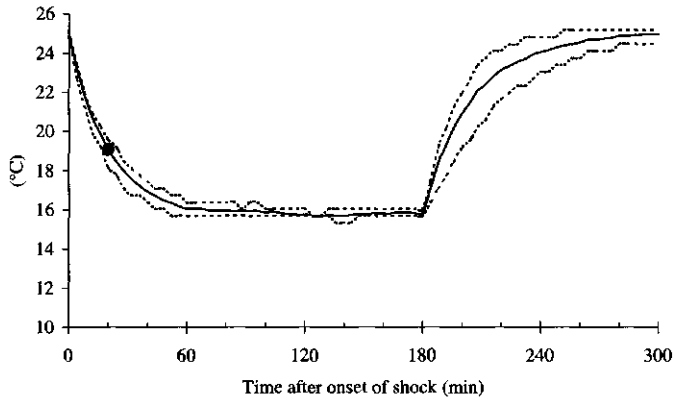

Figure 1: Temperature profile of a 9°C cold shock (solid line) with minimum and maximum values (dotted lines) recorded during the eight different sampling days.

2.7 Plasma and data analysis

Plasma cortisol levels were determined by radioimmunoassay (RIA) (De Man et al., 1980). Plasma glucose was analysed with a GOD/PERID kit (no. 124 028, Boehringer Mannheim GmbH, Mannheim, Germany). Lactate concentrations were analysed using a lactate kit (no. 735-10, Sigma Aldrich, St. Louis, MO, USA).

Genomic DNA, isolated from the blood cell pellets, was used to characterise all sampled androgenetic individuals with 11 microsatellites (see chapter 5). In short, genomic DNA could be isolated from 566 out of the 660 androgenetic individuals sampled. From the 566 individuals characterised, 512 belonging to 33 progeny groups proved to be homozygous at all loci examined. Only data from these 'homozygous' individuals were included in the present data analysis. For each individual, the Fulton condition factor (K) was calculated as

$$K = \frac{100 \times W}{L^3} \qquad (1)$$

where W is the weight (g) and L the length (cm).

2.8 Heritability estimation

Data were analysed using the following statistical model

$$\mathbf{y} = \mathbf{X}\beta + \mathbf{Za} + \mathbf{e} \quad (2)$$

where **y** is a vector with phenotypic observations; **X** is a design matrix relating non-genetic fixed effects to observations; β is a vector with non-genetic fixed effects, **Z** is a design matrix relating additive genetic effects to observations. The distribution of **e** is $N(\mathbf{0}, \mathbf{I}\sigma_e^2)$ where σ_e^2 is the error variance. The covariance structure of the additive genetic effects is expressed as $\mathbf{a} \sim (\mathbf{0}, \mathbf{A}\sigma_a^2)$ where **A** is the additive genetic relationship matrix and σ_a^2 is the additive genetic variance. The additive genetic relation between a sire and its androgenetic progeny as well as the additive genetic relationship among individuals from an androgenetic family is equal to 1 (Bongers et al., 1997b). An ANOVA analysis (SAS 6.12, SAS Institute Inc., 1990) was carried out to determine whether the experimental unit, the aquarium position or sampling day had a significant effect on the recorded phenotypic traits. The results indicated that neither of these had an effect on the traits weight, length, K, glucose and lactate. Therefore, the population mean was the only fixed effect included in the model used to estimate the heritabilities for these traits. For plasma cortisol, a significant ($P < 0.05$) sampling day effect was found. Therefore, the cortisol heritability was estimated using two different models. The first was identical to the model used for the other phenotypic traits. The second model also included sampling day as a fixed effect. The 33 sires and their androgenetic progeny were included in the analysis. Phenotypic observations were available on the progeny (Table 1).

The Gibbs sampler is used to generate samples from a joint density (e.g. Gelfand and Smith, 1990). Based on these samples, the marginal posterior densities for the heritability and the variance components are constructed. In a Gibbs chain, sampling is applied to all unknown parameters in the model. For updating parameter estimates, the procedure as described by Janss et al. (1995) is used. For statistical inference, a long Markov chain is produced by repeating the updating scheme. The consecutive realisations will show serial correlations. To remove correlations, thinning is applied. Further, the Markov chain requires a number of iterations before the equilibrium distribution is reached, i.e. the 'burn in' period. In the present analysis, the thinning parameter was 500 and the burn in period was 250 iterations. For cortisol and lactate, the Gibbs chain was run with a chain length of 2,000,000 cycles leaving 4,000 independent samples that were used to construct posterior distributions for the

parameters. For the other traits, a Gibbs chain of 500,000 iterations was used leaving 1,000 independent samples from the posterior distribution. The posterior distributions were used to determine the posterior mean and the 90% highest posterior density (HPD) region. The 90% HPD region reflects the accuracy of the estimate and can be seen as the Bayesian equivalent of the confidence interval.

Table 1: Number of observations (*n*), mean phenotypic value and standard deviation (SD) for weight, length, condition factor (K), cortisol, glucose and lactate measured in androgenetic individuals subjected to a 9°C cold shock at the age of 110 days post hatching.

Trait	n	Mean	SD
Weight (g)	512	40.9	20.9
Length (cm)	512	10.6	2.0
K ($g\ cm^{-3} \times 100$)	512	3.2	0.6
Cortisol ($nmol\ l^{-1}$)	496	717	248
Glucose ($mmol\ l^{-1}$)	511	1.9	0.8
Lactate ($mmol\ l^{-1}$)	511	1.9	0.6

3 Results

The mean values of the different traits recorded in the androgenetic individuals are shown in Table 1. Results of the heritability estimations are shown in Table 2. Estimates for the morphological traits length and weight were low, but both 90% HPD regions did not include zero. The condition factor (K), had a medium heritability (90% HPD region: 0.20 – 0.62).

The marginal posterior distributions for the plasma traits cortisol, glucose and lactate are shown in Figure 2. These distributions resulted in heritability estimates of different magnitude (Table 2). The mean of the marginal posterior distribution for cortisol was high, but the estimated 90% HPD region was relatively broad (0.37 – 0.9; Figure 2). Including sampling day as fixed effect into the model resulted in a cortisol heritability estimate of 0.31 with a 90% HPD range of 0.15 – 0.56. For glucose, the

estimated heritability was low and the marginal posterior distribution was small (Table 2, Figure 2). For lactate (Figure 2), the heritability estimate was high (0.56).

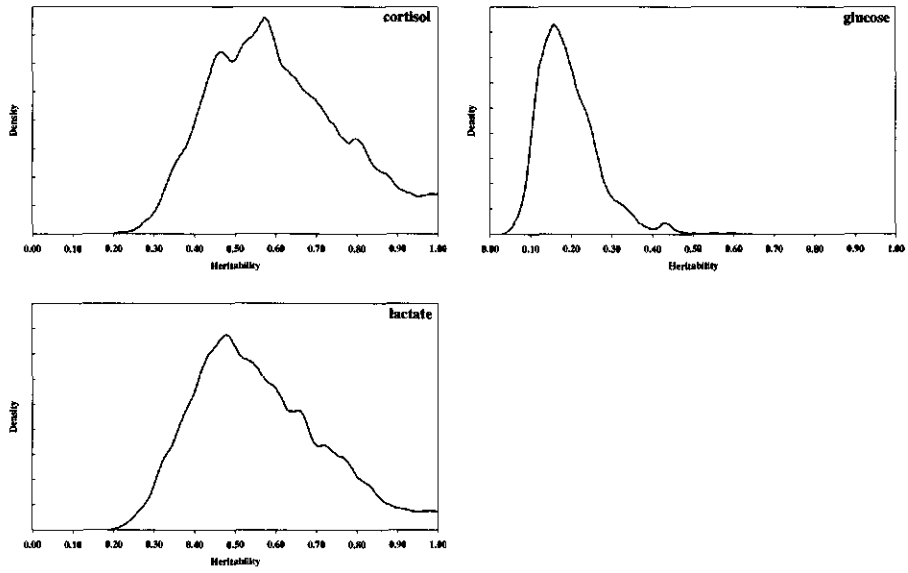

Figure 2: Marginal posterior distribution of heritability estimates for plasma cortisol, glucose and lactate using Gibbs sampling.

Table 2: Estimated additive genetic variance (σ_a^2), error variance (σ_e^2) and mean of marginal posterior distribution of heritability estimates for weight, length, condition factor (K), cortisol, glucose and lactate from androgenetic individuals subjected to a 9°C cold shock at 110 days post hatching. The 90% highest posterior density region is given between brackets.

Trait	σ_a^2	σ_e^2	h^2	
Weight (g)	35.8	379.5	0.09	(0.03 – 0.17)
Length (cm)	0.40	3.15	0.11	(0.04 – 0.21)
K (g cm^{-3} ×100)	0.11	0.18	0.37	(0.20 – 0.62)
Cortisol (nmol l^{-1})	23971	15882	0.60	(0.37 – 0.90)
Glucose (mmol l^{-1})	0.10	0.43	0.19	(0.10 – 0.33)
Lactate (mmol l^{-1})	0.12	0.10	0.56	(0.33 – 0.85)

4 Discussion

4.1 Design

Sampling variances for heritability estimates ($\sigma^2_{h^2}$) in gynogenetic or androgenetic designs are lower than those observed in a conventional half-sib design, when $h^2 < 0.35$ (Bijma et al., 1997). Previously estimated repeatabilities (95% confidence limits: 0.00 – 0.29 and 0.02 – 0.38; 2 lines with n = 60 per line; M.W.T. Tanck, unpublished results) indicated that stress-related cortisol heritabilities in carp would most likely be lower than 0.35. Therefore, we decided to use a large number of androgenetic individuals to estimate heritabilities of stress-related traits in common carp.

Due to the experimental set-up and model used, some environmental effects (aquarium and day of sampling) were confounded with sire (genetic) effects. Because of this, the estimated σ^2_a of these traits might be partly due to genetic effects and partly due to common environmental effects, leading to an overestimation of the heritabilities. However, a bias will only be present when the environmental factor actually affected the trait. Since no significant effect of experimental unit or aquarium position within these units on the traits measured was found, the proportion of variance due to individual aquarium effects is expected to be low or non-existent. There was a significant influence of the sampling day on plasma cortisol levels. However, this day effect was mainly due to higher cortisol levels on only one of the sampling days and, therefore, the possibility that the individuals tested at this day had a genetic predisposition for a high stress response can not be ruled out. Consequently, this day effect might be partly due to genetic and environmental effects and including this day effect in the model possibly resulted in underestimation of the cortisol heritability.

4.2 Heritabilities

Selection among parents would lead to a reduced variance, resulting in an underestimation of the observed heritability (Bulmer, 1971; Falconer and Mackay, 1996). Since mortality can be regarded as a form of (natural) selection, selective mortality within the F_1 sires could influence the heritability estimations, but no data concerning this were available. However, traits recorded in the androgenetic individuals had previously been recorded in the F_1 sires (M.W.T. Tanck, unpublished results). As estimated phenotypic correlations between values recorded in the sires and observed

mortalities in the androgenetic progeny groups were not significant, it can be argued that probably no selective mortality occurred in the F_1 that could have influenced the estimated heritabilities.

Since individual weight and length of the fish are measured routinely in our experiments, heritabilities were also estimated for these traits and the calculated condition factor (K). The estimates for weight (0.09) and length (0.11) at 110 dph are low, but in the range of earlier reported estimates in carp of similar age, weight: 0.10 ± 0.20 (Kirpichnikov, 1972) and 0.12 – 0.48 (Nagy et al., 1980) and length: 0.03 – 0.37 (Aulstad et al., 1972). However, for two reasons heritabilities under commercial practice might be higher. First, the restricted feeding regime applied in the present experiment might have prevented potential genetic differences in individual growth rates coming to full expression. Second, as for gynogenesis (Bongers et al., 1997a), the shock treatment in androgenesis might induce an increased amount of additional environmental variation in morphological traits due to embryonic damage. Despite the low heritability estimates for weight and length, the estimate for K was of medium magnitude (0.37), indicating that this trait is less influenced by the effects of restricted feeding and the environment in general.

The major aim of the present chapter was to estimate heritabilities for stress-related traits. The elevated cortisol concentrations indicate that the fish were stressed at the time of sampling. Consequently, the high cortisol heritability estimate should be regarded as an indicator for a genetic basis underlying the stress response due to a cold shock, whereas the heritabilities for glucose and lactate provide evidence for a genetic basis for basal plasma glucose and lactate levels in carp. The experiments described in chapter 2 showed, that a 9°C cold shock did not have any effect on plasma glucose, and only a slight effect on plasma lactate after 60 minutes. Since that research was carried out with one (isogenic) carp strain only, plasma glucose and lactate levels were again measured in the present experiment. However, both plasma glucose and lactate concentrations found in the present experiment (1.8 ± 1.0 and 1.7 ± 0.8 mmol l^{-1}, respectively) are similar to those found in the experiments described in chapter 2 and can, therefore, be regarded as basal levels for carp held under these conditions. A low heritability for basal glucose (0.025 ± 0.06) has been reported by Del Valle et al. (1996) in unstressed clonal lines of ayu (*Plecoglossus altivelis*). No references for (stressed or basal) plasma lactate heritabilities in fish could be found.

The heritability estimate for cortisol was higher than the previously obtained repeatability estimates (M.W.T. Tanck, unpublished results). Although these repeatability estimates are rather rough, the apparent discrepancy between the cortisol repeatability and heritability estimates is striking. Repeatability, the correlation between repeated measurements of the same individual is equal to $(V_G + V_{E_p}) / V_P$ where V_G = genotypic variance, V_{E_p} = permanent environmental variance and V_P = total phenotypic variance. Consequently, the theoretical heritability of a certain trait can never be larger than the matching repeatability. The repeatabilities were estimated in 180 – 240 days old carp, whereas the androgenetic individuals were 110 days of age. Therefore, the two stress responses might be considered as different traits. Using genetically different populations might also cause a discrepancy. However, the two populations were genetically not that different, since the repeatability was estimated in an F_1 and the heritability in an androgenetic F_2 of the same base population. A more or less similar discrepancy was found in rainbow trout by Fevolden et al. (1991, 1993b and 1999). They found a low correlation ($r = 0.138$) between repeated cortisol measurements, but found cortisol heritabilities of 0.27 ± 0.10 and 0.59 (no SD reported) for juvenile and adult rainbow trout, respectively. Aragaki and Meffert (1998) also found lower repeatabilities than heritabilities, when examining courtship traits in the housefly (*Musca domestica*). They demonstrated that repeatabilities are labile within individuals and may actually underestimate heritabilities when there is a genetic predisposition not to repeat performance (learning). Individual differences in habituation to cold shocks, as reported in chapter 2, might have lead to an underestimation of the cortisol repeatabilities. Social interactions have been reported to both influence individual stress responses (Ejike and Schreck, 1980; Pottinger and Pickering, 1992) and lead to low repeatabilities (Travis and Woodward, 1989).

In conclusion, the androgenetic design used in the present study offers a valuable tool to estimate heritabilities as an integral part of a selective breeding programme aimed at producing homozygous inbred strains. Although the level of the reported cortisol heritability has to be regarded with some reservation due to the experimental design, the heritability clearly shows that the stress response due to a cold shock is hereditary in the carp population used. Therefore, individuals from this population with either a high or low breeding value for stress will be selected and used to create homozygous inbred or F_1 hybrid strains with divergent stress responses. These strains can be utilised to further study the stress response and its effects on important traits, like growth, maturation, disease resistance and animal welfare in general.

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Chapter 5

Segregation of microsatellite alleles and residual heterozygosity at single loci in homozygous androgenetic common carp (*Cyprinus carpio* L.)

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Genome, submitted

Abstract

Thirty-three androgenetic progeny groups of common carp were analysed using 11 microsatellite markers to: 1) verify the homozygous status of the 566 androgenetic individuals, 2) analyse the microsatellite allele segregation, and 3) study possible association of microsatellite alleles with phenotypic traits. In total, 92% of the androgenetic individuals proved to be homozygous at all 11 loci. Forty-three out of the 47 heterozygous individuals were heterozygous at a single locus only. This heterozygosity was probably due to DNA fragments caused by UV-irradiation of the eggs, although the maternal origin of the fragments could not be proved beyond doubt. Screening with 11 microsatellites also revealed two linkage groups, a segregation distortion at two microsatellite loci and possible association of some microsatellites with weight, length, stress-related plasma cortisol levels and basal plasma glucose levels. The success of the linkage and association study could be explained by a low recombination frequency due to high chiasma interference. This would imply a relatively short genetic map length for common carp.

1 Introduction

Segregation of microsatellite alleles can be examined in a cross between lines, but haploid progenies provide a more powerful tool for segregation and linkage analysis, due to the absence of problems associated with heterozygosity and diploidy (Lie et al., 1994; Slettan et al., 1997; Spruell et al., 1999). However, since only few phenotypic traits can be measured on haploids, the use of doubled haploids (DH) is more useful for detecting QTLs associated with e.g. growth, maturation and disease resistance (Streisinger et al., 1981; Kocher et al., 1998). The fact that DH lines can be maintained indefinitely is an additional advantage in marker and trait analyses (Burr et al., 1988). Although DH lines were used to create a linkage map in rainbow trout, *Oncorhynchus mykiss* (Young et al., 1998), relatively few reports using DH lines for segregation studies in fish are available compared with e.g. plants, where this is a more common practice (e.g. Foisset and Delourme, 1996).

In an earlier study, six full-sib families, with five or six androgenetic progeny groups each, were used to estimate heritabilities for morphological and stress-related traits in carp, *Cyprinus carpio* L. (chapter 4). Since the homozygosity of the androgenetic individuals was an essential assumption underlying the model used to estimate the variance components, all androgenetic individuals were tested at 11 microsatellite loci. In this paper, we present a detailed analysis of this homozygosity testing. In addition, we used the microsatellite genotyping data of the homozygous

individuals to analyse the segregation at the different loci and to test for possible linkage between microsatellite markers. Furthermore, using the phenotypic traits recorded previously, the association between markers and these traits was studied.

2 Material and Methods

2.1 Fish

Six full-sib families (E4×AP1-6, Figure 1), were produced by crossing six wild outbred AP grandsires with one domesticated homozygous E4 granddam (see chapter 3). Per full-sib family, five to six sires were randomly selected for androgenetic reproduction. In a total of 8 sessions, 33 androgenetic progeny groups were produced. Androgenesis was performed using the method described by Bongers et al. (1994). For each sire, a total of 5 egg batches, each containing approximately 200 eggs in 5 ml synthetic ovarian fluid, were irradiated in a petridish with a total UV-dose of 175 mJ cm^{-2} (λ: 254 nm) while gently stirring the eggs. Thereafter, the eggs were fertilised with 200 µl of milt and placed in the incubation system (24°C). Thirty minutes after fertilisation, a 2 min heat shock was administered by transferring the eggs from the incubation system to a water bath of 40°C. This timing corresponds to the metaphase of the first mitotic division of the zygotes (Komen et al., 1991; Bongers et al., 1994). In each session, appropriate numbers of egg and sperm quality controls (non-irradiated eggs fertilised with normal sperm: diploids) and irradiation-controls (irradiated eggs fertilised with normal sperm, but no heat shock: haploids) were taken along. As egg-donor the standard E4E5 strain was used, because of its good egg quality. This strain was produced by crossing a homozygous E4 female with a spontaneously sex-reversed homozygous E5 male (x/x mas/mas) (Komen et al., 1991). E5 alleles differ from E4 alleles at approximately 50% of the microsatellites developed so far.

Each androgenetic progeny group was reared separately in a 140 l aquarium connected to a recirculation system consisting of a plate separator, a biological reactor and an UV treatment unit. The rearing temperature was 25°C. Until 21 days post hatching (dph), fish were fed freshly hatched *artemia* nauplii. Thereafter, fish were fed a commercial trout pellet (Provimi, 91 series, Rotterdam, The Netherlands). Per progeny group, the survival percentages in the different rearing periods (0-21 and 22-110 dph) were calculated based on counts of surviving fish at 0, 21 and 110 dph.

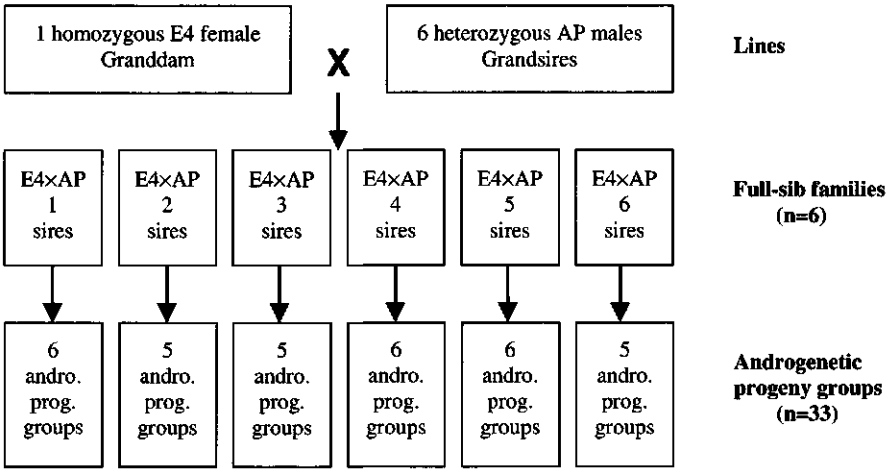

Figure 1: Schematic representation of the pedigree structure in the carp population used. Andro. prog. groups: Androgenetic progeny groups.

2.2 Sampling, DNA isolation and genotyping

All 33 androgenetic progeny groups were subjected to a 9°C cold shock and blood was sampled 20 min after onset of the shock at an age of 110 dph (chapter 4). Blood samples were taken from maximal 20 (range 13 – 20) randomly picked individuals per androgenetic progeny group by puncturing the caudal vessels with a 1 ml syringe containing Na_2-EDTA as anti-coagulant. After centrifugation, blood cell pellets were stored at -20°C pending DNA isolation. From each sampled individual, the phenotypic traits weight, (standard) length, stress-related plasma cortisol concentration, and basal plasma glucose and lactate concentrations were recorded.

Genomic DNA was isolated using the Puregene DNA isolation kit (Gentra systems Inc., NC, USA). Each individual fish was genotyped with 11 microsatellite markers (Microsatellite Fish Wageningen, MFW). Per fluorescence dye, the microsatellites were selected based on allele lengths and level of heterozygosity displayed in the 33 sires used (chapter 3). The selected microsatellites were: MFW001, 002, 009 and 028 (described by Crooijmans et al., 1997) and MFW035, 040, 046, 048, 050, 059 and 071 (Table 1). PCR was carried out as described by Crooijmans et al. (1996) and all 11 MFWs were combined in one multiplex set. This mixture was separated on a 6% denaturing polyacrylamide gel (Sequagel-6, National Diagnostics, Atlanta, GA, USA) using an ABI 373 automated sequencer (Perkin Elmer, Foster City,

CA, USA). Gels were interpreted using Genescan and Genotyper software packages (Perkin Elmer).

Table 1: Primer sequences and repeats of MFW035, 040, 046, 048, 050, 059 and 071. A PCR annealing temperature of 55°C was used for all MFWs.

MFW	Primer sequence*	Repeat
MFW035	FCTGTCTGTGAGATGTAGAGAG	$(TG)_8CG(TG)_{21}$
	CCAAAATGACCTCCTGTTCTC	
MFW040	FCACGTCTCATCTACTCAG	$(CA)_{41}$
	CTGGTGACCGATCCATTTATGAG	
MFW046	FGCTTATGCTCACCAAGGAAAC	$(TA)_{29}N_8(CA)_9N_6(TA)_{11}$
	GGTACAGAAAAGACACTGGA	
MFW048	FCTCCTGACCACATATACATGC	$(TG)_{10}T(TG)_3T(TG)_7T(TG)_9$
	CTTGGAAGTCAGCTCCCTATG	
MFW050	FGGGGCAAAAACAAGTGTTGCG	$(TG)_{19}$
	GCGTCAGGTAGGCTAAGTCAAG	
MFW059	FGATGTTTGTTGTATGCCCAC	$(TG)_{44}$
	GTGTCATTCCTGTTATGCAG	
MFW071	FGTGCACTTATCAGTCTCAGTG	$(AC)_{20}$
	CGAAAGATAAGCGGTGCACT	

* The primer sequence was written in the 5' to 3' direction, where the first primer is fluorescent labelled with fluorprime (F) (Pharmacia Biotech, Uppsala, Sweden)

2.3 Survival

The effect of grandsire on survival percentages was tested by ANOVA (Proc GLM, SAS 6.12, SAS Institute Inc., 1990) using the model:

$$Y_{ij} = \mu + GS_i + e_{ij} \quad (1)$$

where Y_{ij} = the surviving % of androgenetic progeny, j = 1,..., 6 (maximum number of sires within grandsires), μ = the overall mean, GS_i = the effect of grandsire (i=1, ..., 6) and e_{ij} = the residual error term. Residues of the model were normally distributed (Wilk-Shapiro, W > 0.90) and had equal variances between grandsires (Levene's test), making

transformation unnecessary. *P*-values < 0.05 were regarded as significant. Values in the text are expressed as mean ± SD.

2.4 Verification of homozygous status

Genotypes from the E4 granddam and the 33 E4×AP sires were used to reconstruct the genotypes of the six AP grandsires. Per androgenetic progeny group, the percentage of homozygous animals was calculated. Animals were considered homozygous if they were homozygous at all 11 microsatellite loci. The data was examined for a possible correlation between the percentage of homozygous animals and the percentage of normal larvae in the irradiation controls. Since these normal larvae are heterozygous due to incomplete inactivation of the female genome during irradiation, the correlation is expected to be negative.

2.5 Segregation of microsatellite alleles

Only homozygous individuals in the androgenetic progeny groups were used in the analysis of allele segregation, the linkage analysis and in the association study. Segregation was studied in the total population and per separate E4×AP full-sib family by scoring the number of E4 and AP alleles. The latter analysis was carried out to examine for possible differences in segregation between full-sib families, which might indicate differences in the microsatellites alleles and genes situated in the adjacent DNA, who could influence fitness. Segregation was tested for deviations from Mendelian 1:1 segregation (using a χ^2-test with 1 degree of freedom). A Bonferroni correction for multiple tests was performed to adjust the threshold ($P < 0.05$) for the number of tests. This resulted in a critical value for χ^2 of 8.01 (11 comparisons) for the population analysis and a critical value of 11.29 (66 comparisons) for the analysis of separate full-sib families.

2.6 Linkage between microsatellites

Per microsatellite combination, only those homozygous animals whose sires were informative at both loci were included in the linkage analysis. LOD scores were calculated to assess linkage. A LOD threshold of 3.0 was interpreted as significant linkage and a LOD score between 2.0 and 3.0 was considered suggestive linkage (Botstein et al., 1980). A 90% confidence interval was calculated for the estimated percentage of recombinants using the 'one-LOD-drop' method.

2.7 Association between microsatellites and quantitative traits

Association of microsatellites markers with the phenotypic traits weight, length, and plasma cortisol, glucose and lactate levels was studied using two models.

2.7.1 *Line-cross model*

The first model is a line-cross analysis. This model is aimed to pick up QTLs that cause a difference for the phenotypic traits between the E4 granddam and the AP grandsires, assuming that the two founder lines E4 and AP have different alleles at the QTL affecting the phenotypic trait of interest, although the granddam and grandsires might share alleles at the microsatellite locus under investigation. This assumption is based on the different backgrounds of E4 and AP (domesticated vs. wild). The analysis was carried out using the model:

$$Y_{ijk} = \mu + S_i + M_j + e_{ijk} \quad (2)$$

where Y_{ijk} = the observed value, μ = experimental mean, S_i = random effect of sire (i = 1,..., 33), M_j = effect of maternal or paternal microsatellite allele (j = E4 or AP) and e_{ijk} = residual error term.

2.7.2 *Full-sib model*

The second model is an analysis nested within the full-sib families. This model can pick up QTLs that are linked to the genetic markers, assuming differences between individual AP grandsires and the E4 granddam. This analysis was carried out using the following ANOVA model:

$$Y_{ijkl} = \mu + S_i + M_j(EA_k) + e_{ijkl} \quad (3)$$

where M_j is nested within the full-sib families (EA; k = 1,...,6). Residues of both models were normally distributed (Wilk-Shapiro test; W > 0.90) and variances were equally distributed (Levene's test).

2.7.3 *Significance thresholds*

Two significance levels were defined. After Bonferroni correction for the multiple F-tests executed, the comparisonwise threshold was $P < 0.0009$ (55 comparisons and an overall P-value of 0.05) in the line-cross model and $P < 0.0002$ (330 comparisons) in the full-sib model. The second level is suggestive association,

where overall one false positive is accepted (similar to Lander and Kruglyak, 1995). For the line-cross model, this threshold is $P < 0.018$ and for the full-sib model $P < 0.003$.

3 Results

The genotypes of the E4 granddam and AP grandsires (reconstructed) for the 11 microsatellite loci used are shown in Table 2. The mean number of alleles observed at the individual microsatellite loci was 5.3 ± 1.5.

Table 2: Microsatellite alleles (in base pairs) found in the E4 granddam and the six AP grandsires at the 11 MFW loci studied.

MFW	E4	AP1	AP2	AP3	AP4	AP5	AP6
001	224	170 / 210	210 / 218	170 / 221	210 / 221	170 / 218	170 / 170
002	253	256 / 275	256 / 260	260 / 275	246 / 256	265 / 275	250 / 253
009	120	120 / 123	123 / 138	123 / 123	136 / 138	123 / 136	123 / 128
028	301	295 / 301	286 / 295	295 / 295	295 / 295	295 / 295	295 / 295
035	219	217 / 217	215 / 224	217 / 221	224 / 224	221 / 224	210 / 224
040	218	197 / 227	197 / 218	214 / 227	184 / 184	184 / 188	188 / 193
046	306	290 / 290	290 / 290	290 / 290	290 / 290	290 / 290	290 / 300
048	384	379 / 398	379 / 384	371 / 375	384 / 390	379 / 398	375 / 379
050	386	327 / 381	327 / 381	321 / 325	323 / 381	319 / 327	325 / 327
059	295	285 / 290	279 / 279	279 / 279	285 / 290	275 / 290	279 / 285
071	119	119 / 136	130 / 136	121 / 136	136 / 136	136 / 136	130 / 136

Note: E5 alleles are equal to E4 alleles except at MFW009: 186 bp; MFW035: 224 bp; MFW040: 192 bp and MFW059: 245 bp.

3.1 Verification of homozygous status

The mean yield (± SD) of diploid androgenetic larvae in the different sessions was 13.9 ± 2.3%. In the irradiation controls, the mean percentage of normal diploid larvae was 0.8 ± 0.9%. The mean survival rate within the different androgenetic progeny groups was 47.4 ± 22.5% between 0 and 21 dph and 84.2 ± 10.6% between 22 and 110 dph. A significant lower mean survival percentage between 0 and 21 dph was observed in androgenetic progeny groups from the AP3 grandsire (21.8 ± 13.9%)

Table 3: Segregation of microsatellite alleles at MFW009, 028 and 048. Per full-sib family, the two AP grandsire alleles, the number of sires with a particular grandsire allele, and the number of androgenetic individuals within these sires with either a paternal AP or maternal E4 allele are given (ni = non-informative sires). The segregation was analysed by χ^2-test (1 df; $P < 0.05$) in the population (Pop.) and within the six E4×AP full-sib families. For comparisonwise critical values of χ^2 see materials and methods section. The number of heterozygous androgenetic individuals (#Het) within the different classes is also shown.

MFW009	E4×AP1		E4×AP2		E4×AP3		E4×AP4		E4×AP5		E4×AP6		Pop.
E4: 120/120	120	123	123	138	123	123	136	138	123	136	123	128	
# sires	3	3	1	4	5		5	1	3	3	3	2	
Paternal AP	ni	9	4	39	45		8	13	14	9	19	14	174
Maternal E4	ni	13	5	28	22		64	5	22	21	26	18	224
#Het.	ni	0	0	0	0		1	0	0	0	0	0	1
χ^2	0.73 ns		1.32 ns		7.90 ns		25.60 *		6.06 ns		1.57 ns		6.28 ns

MFW028	E4×AP1		E4×AP2		E4×AP3		E4×AP4		E4×AP5		E4×AP6		Pop.
E4: 301/301	295	301	286	295	295	295	295	295	295	295	295	295	
# sires	5	1	1	4	5		6		6		5		
Paternal AP	38	ni	8	34	32		39		39		30		220
Maternal E4	27	ni	2	30	36		25		34		23		177
#Het.	4	ni	0	1	1		3		5		8		22
χ^2	1.86 ns		1.35 ns		0.24 ns		3.06 ns		0.34 ns		0.92 ns		4.6 ns

MFW048	E4×AP1		E4×AP2		E4×AP3		E4×AP4		E4×AP5		E4×AP6		Pop.
E4: 384/384	379	398	379	384	371	375	384	390	379	398	375	379	
# sires	5	1	3	2	4	1	3	3	3	3	1	4	
Paternal AP	26	9	12	ni	28	8	ni	11	12	27	6	14	153
Maternal E4	43	3	34	ni	30	6	ni	20	26	20	10	21	213
#Het.	0	0	0	ni	0	0	ni	0	1	0	0	0	1
χ^2	1.49 ns		10.5 ns		0.00 ns		2.61 ns		0.58 ns		2.37 ns		9.84 *

compared to androgenetic progeny groups from the AP5 grandsire (60.5 ± 21.7%) and AP6 grandsire (65.6 ± 16.0%). There was no effect of grandsires on the survival percentages in the period between 21 and 110 dph.

Of the 640 fish sampled at 110 dph, DNA could be isolated from 566 individuals. Of these fish, 519 proved to be homozygous at all microsatellites loci studied. The mean percentage of homozygous fish per androgenetic progeny group was 92 ± 8%. There was no significant negative correlation between the percentage of diploid larvae in the UV-irradiation controls at a particular session and the matching percentage of homozygous fish. Of the 47 heterozygous fish, 43 fish were heterozygous at only one microsatellite locus, two were heterozygous at two loci and the remaining two individuals were heterozygous at three and five loci, respectively. At all 11 microsatellite loci, some heterozygous individuals were found. The mean percentage of heterozygous individuals per locus was 1 ± 1%, with 4.7% at MFW028 as highest value (Table 3). Of the 11 MFWs used, the egg-donor E4E5 had distinguishable E5 alleles at MFW009, 035, 040 and 059 (Table 2). In total, nine heterozygous fish were found at these four loci of which two had a sire with an identical allele as E5. In the remaining seven heterozygous fish, no maternal E5 alleles were found.

3.2 Segregation of microsatellite alleles

On the population level, one significant deviation from Mendelian expectation was observed at microsatellite locus MFW048 (Table 3). On the full-sib family level, MFW009 showed a significant distortion in the E4×AP4 family. In both cases, an excess of maternal E4 alleles was found.

3.3 Linkage between microsatellites

Two pairs of microsatellite loci showed linkage with a LOD score larger than 3 (Table 4). The other combinations all showed LOD scores lower than 2.

3.4 Association between microsatellites and quantitative traits

The results of the ANOVA using the line-cross model (2) are shown in Table 5. No significant associations were found. The associations of MFW001 with glucose and MFW050 with weight and length are suggestive. MFW028, which is closely linked with MFW001, was not associated with glucose ($P = 0.255$).

Table 4: Linkages found between carp microsatellite loci. Per combination the total number of individuals with a particular haplotype, the Log of Odds-ratio (LOD) score, the percentage of recombinants (θ) and the 90% confidence interval (90% c.i.) are given. The 90% c.i. were estimated using the 'One-LOD-drop' method.

Loci	Observed progeny haplotypes				LOD score	θ	90% c.i.
	AP-AP	AP-E4	E4-AP	E4-E4			
MFW001 & 028	184	9	3	135	77	3.6	(1.9 – 6.3)
MFW035 & 040	137	30	27	135	33	17.3	(13.1 –22.1)

Table 5: Results of the ANOVA using model 2, which tested for differences in weight, length, cortisol, glucose or lactate between individuals with either a maternal E4 or paternal AP allele at a particular microsatellite. Only traits with a *P*-value < 0.05 for the model are shown.

MFW	Trait	P-value	Least square means	
			E4	AP
1	Glucose	0.009*	1.8 mmol.l^{-1}	2.0 mmol.l^{-1}
2	Glucose	0.023	2.0 mmol.l^{-1}	1.8 mmol.l^{-1}
9	none	--	--	--
28	none	--	--	--
35	none	--	--	--
40	none	--	--	--
46	none	--	--	--
48	none	--	--	--
50	Weight	0.011*	37.4 g	42.7 g
	Length	0.005*	10.3 cm	10.8 cm
59	none	--	--	--
71	none	--	--	--

*: suggestive association; **: significant association

Analysis within E4×AP full-sib families using model 3 are shown in Table 6. In none of the full-sib families a significant association was found. Only MFW040 showed suggestive association with cortisol in the E4×AP2 family.

Table 6: MFW with significant association with quantitative traits analysed per separate E4×AP full-sib family (ANOVA; Model 3). The *P*-values are given between brackets.

Full-sib family	Trait				
	Weight	Length	Cortisol	Glucose	Lactate
E4×AP1				40 (0.004) 46 (0.040)	
E4×AP2	2 (0.020) 46 (0.037)	2 (0.014)	40 (0.0008)*	35 (0.042)	
E4×AP3	35 (0.026)				2 (0.019)
E4×AP4	50 (0.019)	50 (0.020)	35 (0.040)	50 (0.032)	
E4×AP5			40 (0.024)	1 (0.025)	
E4×AP6			50 (0.026)	1 (0.023) 28 (0.029)	

*: suggestive association; **: significant association

4 Discussion

4.1 Verification of homozygous status

The observed percentage of heterozygous individuals (8%) was higher than expected given the relative low percentage of diploid larvae found in the UV-controls (< 1%). Androgenetically produced fish larvae of different species have been screened previously for possible maternal contamination and residual heterozygosity using either colour markers (Bongers et al., 1994; Rothbard et al., 1999), DNA content measures (Lin and Dabrowski, 1998), enzyme markers (Scheerer et al., 1991) or DNA markers like fingerprinting (Young et al., 1996; Sarder et al., 1999) and microsatellites (Corley-Smith et al., 1996). Although the majority of the results presented in those studies

suggest successful androgenetic reproduction, Lin and Dabrowski (1998) and Rothbard et al. (1999) also found some heterozygous individuals in androgenetic progeny groups. Possible causes could be insufficient irradiation, resulting in eggs with intact maternal DNA, or participation of maternal chromosome fragments in mitotic division (Grunina et al., 1990; Lin and Dabrowski, 1998). If complete or fragmented maternal DNA would be present in the androgenetic individuals, this resulted in individuals carrying a maternal E4 or E5 allele and a paternal E4 or AP. In case the animals received both E4 alleles, they are homozygous and cannot be distinguished from truly androgenetic individuals. If animals received a distinguishable E5 allele, the presence of maternal DNA is irrefutable. However, E5 alleles are only different from E4 alleles at four of the 11 MFWs used, and no specific E5 alleles were found in any of the heterozygous fish. If both the E4 and AP allele are present, this could be indicative of maternal contamination. Based on allele patterns of the egg-donor and the 33 E4×AP sires, the average chance per sire to detect maternal contamination at a single microsatellite locus is 56% (Range 39-75%). In case the complete maternal genome was inherited (or fragments carrying all used microsatellite loci), the average probability to detect maternal contamination based on nine (unlinked) loci is 99.9%, and the androgenetic individuals would most probably be heterozygous at more than one microsatellite locus. However, the majority of the 47 heterozygous individuals was heterozygous at one locus only and MFW028 was the most abundant heterozygous locus with 22 heterozygous individuals. In case the complete maternal genome was inherited, the average chance of finding an individual that is heterozygous at e.g. MFW028 and homozygous at the other loci is 0.05%.

Similar to the occurrence of heterozygous fish in gynogenetic progenies due to spontaneous diploidisation of eggs (Gomelskiy and Rekubratskiy, 1990; Cherfas et al., 1995), diploid sperm could be another possible reason for heterozygous androgenetic offspring. However, if diploid sperm would cause the occurrence of heterozygous fish, one would again expect to find fish that were heterozygous at a number of loci. Furthermore, although diploid sperm was observed in two hybrids of cyprinid species by Cherfas et al. (1994) and Alves et al. (1999), reports about diploid or aneuploid sperm in cyprinids are rare.

The most plausible explanation for the occurrence of animals that were heterozygous at a single locus is the presence of (maternal) chromosome fragments produced during UV-irradiation of the eggs. Studies in plants have shown, that UV-irradiation can also be used to create donor protoplasts containing fragmented DNA instead of the commonly used gamma- or X-rays. These UV-irradiated protoplasts are

subsequently fused with normal recipient protoplasts from a different species to create asymmetric plant hybrids (Vlahova et al., 1997; Forsberg et al., 1998). Furthermore, Forsberg et al. (1998) showed that the amount of donor RFLP loci retained in the hybrid plants decreased with increasing UV-irradiation dose (range: $0 - 468$ mJ cm^{-2}) and that elimination of donor RFLP loci to a large extent resulted from chromosome fragmentation rather than whole chromosome loss. The fact that in the present study the majority of the animals was heterozygous at MFW028, is, to some extent, contrary to the results of Forsberg et al. (1998), who did not find preferential elimination of specific chromosome fragments. The fact that only one individual was heterozygous at both MFW001 and MFW028, despite the close linkage between these loci, would imply that the retained chromosome fragments are relatively small.

Contamination of androgenetic fish with (probably maternal) chromosome fragments caused by UV-irradiation of eggs has been reported by Arai et al. (1992) in loach (*Misgurnis anguillicaudatus*) and Lin and Dabrowski (1998) in muskellunge (*Esox masquinongy*). Although, the maternal origin of the DNA fragments could not be proved in the present study due to the relatedness of the egg-donor and the sires, results indicate that UV-irradiation of eggs can lead to fragmented DNA, and consequently, to some (partly) heterozygous animals in androgenetic progenies. Therefore, androgenetic individuals should be screened thoroughly for the presence of egg-donor DNA fragments, especially when they are to be used as broodstock.

4.2 Segregation of microsatellite alleles

Segregation distortion was observed both at the population level (MFW048) and the full-sib family level (MFW009). Segregation distortion has been observed at microsatellite loci in other fish species e.g. rainbow trout (Young et al., 1998), Chinook salmon *O. tschawytscha* (Banks et al., 1999) and pink salmon *O. gorbuscha* (Spruell et al., 1999). Reasons for segregation distortion at a microsatellite locus can be found both in the PCR-based technique used and in genetic mechanisms. Preferential amplification of the shorter allele in heterozygous animals results in a disproportionate presence of the shorter allele in a population ('drop out' alleles). However, in case of androgenetic reproduction, offspring only receive one allele, thus evading the problem of preferential amplification. A prezygotic genetic mechanism like genic or chromosomal meiotic drive could also explain the segregation distortions found (Lyttle, 1991). This possible cause should be examined further by analysing segregation patterns at linked loci, but

no information about loci that are linked with either MFW009 or 048 is available at the moment.

The most likely cause might be association of a microsatellite locus with a locus or loci influencing e.g. sperm functionality (prezygotic) or larval viability (postzygotic), with complete lethal genes as the most extreme form. The fact that at both MFW009 and 048 generally both alleles were found, indicates that a complete lethal recessive gene was not present in the direct vicinity of these microsatellite loci. However, there was a bias towards the maternal allele. The granddam E4 is a homozygous fish theoretically free of recessive lethal genes, whereas the AP grandsires were derived from a wild population (chapter 3) and are likely to carry a number of alleles associated with reduced survival ('genetic load'). The higher mortality observed in three out of five E4×AP3 progeny groups might be caused by (a) recessive lethal gene(s) carried by the AP3 grandsire based on the inheritance pattern. However, no association between this mortality and any of the 11 microsatellites used was found.

4.3 Linkage between microsatellites

Given the low number of microsatellites used in the present study, the discovery of two linkage groups (MFW001 & 028 and MFW035 & 040) seems rather surprising. Since no microsatellite linkage map for carp is available, the present recombination percentages cannot be compared with previously found results.

4.4 Association between microsatellites and quantitative traits

The present experimental set-up allowed a limited association study. The seven microsatellites and two linkage groups only cover a small part of the genome, considering the haploid chromosome number of 50 in common carp. However, similar to the linkage map, no association studies in carp have been reported so far. As shown in Tables 5 and 6, some significant differences were found in the quantitative traits for carps carrying either the E4 or AP allele at a particular locus. Although no association remained significant after Bonferroni correction, the associations found between MFW001 and glucose and between MFW050 and weight and length in the total population, and the association between cortisol and MFW040 found in the E4×AP2 family, can be considered at least as suggestive.

The fact that, with only 11 microsatellites, two linkage groups and suggestive association of some microsatellite markers with different phenotypic traits were found,

suggests a relatively low recombination frequency in common carp chromosomes. Relative high numbers of linked markers compared to the total number of markers used have also been reported in other studies in fish. Jackson et al. (1998) found seven pairs of linked markers within 24 markers tested in rainbow trout, whereas Slettan et al. (1997) found three linked markers within a total of 13 markers used in Atlantic salmon (*Salmo salar*). The existence of single obligatory chiasmata with high chiasma interference has been reported in various fish species (common carp (Cherfas, 1977, Komen, 1990), plaice, *Pleuronectes platessa* (Purdom et al., 1976; Thompson et al., 1981), zebrafish, *Danio rerio* (Streisinger et al., 1986) and rainbow trout (Thorgaard et al., 1983; Thompson and Scott,, 1984)). High interference thus appears to be more common in fish than in other animals and plants, possibly due to the relatively small size of fish chromosomes (Thorgaard et al., 1983). Low recombination frequencies due to high interference would result in a relative small genetic map length for common carp, and few markers would be needed per chromosome to follow the segregation of QTLs located on these chromosomes. On the other hand, the average physical distance between the genes and co-segregating markers will be large compared to species with a higher recombination incidence per chromosome, making positional cloning of genes in common carp more difficult.

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Chapter 6

Exploring the genetic background of the stress response using isogenic progenies of common carp selected for high or low stress-related cortisol response

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Abstract

Four homozygous (HomIso) and eight heterozygous isogenic (HetIso) strains of common carp were used in two separate experiments to examine the genetic background of a stress-related cortisol response due to a rapid temperature decrease. The homozygous sires (two high and two low) and dams (high and low) used to obtain the strains were selected based on their estimated breeding value for this trait at an age of 15 months (EBV_{15}). In both experiments, the strains were subjected to a 9°C cold shock at an age of 5 months. The ranking in plasma cortisol levels of the HomIso strains was identical to the ranking in EBV_{15} of the sires and the maximal difference of 350 nmol l^{-1} was similar to the expected difference based on these EBV_{15}'s. The mean (±SD) coefficient of variation per strain within an aquarium due to V_E only was 26.6 ± 7.4%. The mean CV between replicate aquaria of a strain was 15.6 ± 2.9%. Differences between the HetIso strains were smaller than expected, and influence of non-additive genetic effects could not be detected ($P_{D\times S} = 0.14$). Estimated breeding values based on the performance of the androgenetic progeny (EBV_5) in experiment 1 and general combining abilities (GCA) of the sires and dams calculated in experiment 2 were positively correlated with the EBV_{15} (r not significantly different from 1), providing no evidence that the stress responses at 5 and 15 months are different traits. Based on the results of these experiments, it can be argued that the best method to change the stress responsiveness of common carp is through selective breeding (exploiting additive genetic effects) rather than through crossbreeding (exploiting non-additive genetic effects).

1 Introduction

Androgenesis and gynogenesis are reproduction techniques, which can be used as powerful tools in the analysis of genetically complex traits. Androgenesis and gynogenesis produce doubled haploid progeny groups (DHG's) from which homozygous sires and dams can be selected to produce (by the same technique) genetically identical progenies and F1 hybrids (Bongers et al., 1998) in only two generations. Since dominance variation (V_D) is absent and assuming that epistatic variation (V_I) is negligible in DHG's, the total genetic variance (V_G) within a DHG only reflects additive genetic variation (V_A), which equals the V_A between DHG's. As a result, the total amount of V_A is doubled after gynogenetic or androgenetic reproduction compared to the V_A in the outbred population the dams or sires originated from (Bijma

et al., 1997; Bongers et al., 1997). Due to this doubling, DHG's are especially useful to estimate heritabilities (h^2) for traits with a low expected heritability (< 0.35).

Variation within androgenetic or gynogenetic homozygous isogenic strains, produced from single DH individuals, only reflects the magnitude of environmental variation (V_E) for a particular trait due to absence of V_A. Although this residual variation can still be large (Komen et al., 1993, Taniguchi et al., 1994), theoretically the mean performance of such a strain should equal the breeding value of the DH sire or dam from which the strain was derived. Finally, crossing of DH dams and sires produces panels of heterozygous isogenic strains, which can be used to examine non-additive genetic (e.g. dominance) effects.

The stress response is a complex trait, consisting of a variety of adaptive physiological and behavioural responses. In fish, stress results in the activation of adrenergic and corticoid pathways similar to that of higher vertebrates. Experiments have revealed that a genetic basis for stress-related cortisol release exists in different salmonid fish species and several selective breeding programmes for this trait have been carried out (Fevolden et al., 1993, 1999; Pottinger and Carrick, 1999a,b). Although common carp (*Cyprinus carpio* L.) is an important species for both aquaculture and research, no selective breeding programmes aimed at producing carp strains with divergent stress-related cortisol responses have been initiated so far.

Common carp has a long history of domestication (Balon, 1995). Under uncontrolled conditions, domestication generally leads to a decrease in genetic variability within a population (Krieg and Guyomard, 1985; Agnèse et al., 1995), while the response to selection is positively related to the magnitude of the genetic variability within a population (Gjedrem, 1983). On the other hand, domestication is known to directly affect the cortisol stress response, as domesticated individuals generally respond less profound when subjected to a stressor than wild individuals (Caldwell Woodward and Strange, 1987; Künzl and Sachser, 1999). It can be expected that due to domestication selection the stress response of common carp has changed considerably.

In a previous study, we estimated the h^2 of a cortisol response to a cold shock in 33 androgenetic DHG's of common carp. The base population consisted of an F_1 between domesticated and feral carp, as we expected that crossing a wild strain with a domesticated strain would increase the genetic variability in the stress response in the (androgenetic) F_2. The estimated h^2 was high (0.6; chapter 4), but has to be regarded with some reservation due to confounding of some environmental effects (tank and sampling day) with sire effects.

In the present study, we investigated the stress response in homozygous and heterozygous isogenic strains derived from androgenetic DH sires and dams. Selection of sires and dams was based on their estimated breeding value for a cold shock induced cortisol response obtained at 15 months of age (EBV_{15}). In a first experiment, we subjected four homozygous isogenic (HomIso) strains to a cold shock at an age of *c.* 5 months to determine whether mean cortisol responses corresponded with the EBV_{15}'s of the sires. In a second experiment, the magnitude of non-additive genetic effects on the cortisol response was investigated. Homozygous sires and dams with known EBV_{15}'s were crossed to produce heterozygous isogenic strains (HetIso; technically known as F_1 hybrid strains; Festing 1979), which were subjected to the same standardised stressor as used in the first experiment. As secondary aims of the experiments, the variation due to environmental sources and possible differences in stress response patterns between strains were examined.

2 Materials and methods

2.1 Selection of androgenetic DH parents

An F_1, consisting of six full-sib families, was created by crossing a homozygous domesticated E4 great-granddam with six wild outbred AP great-grandsires (Figure 1) (described in chapter 3). From each full-sib family, five or six grandsires were randomly picked to produce, in total, 33 double haploid progeny groups (DHG's). At 4 months, the 33 DHG's (F_2) were subjected to a standard stressor: a 9°C cold shock and blood sampled 20 min after onset of the shock (described in chapter 4).

Selection of individual fish from the 33 DHG's based on the response at 4 months was not possible, due to the fact that the animals could not be individually tagged. Therefore, three DHG's with a high mean plasma cortisol concentration (H1-3) and three DHG's with a low mean plasma cortisol concentration (L1-3) were selected (Table 1). The 154 fish (mean body weight ± SD: 349 ± 168 g) in these six DHG's were individually tagged (Tagscan, Alphen a/d Rijn, The Netherlands), mixed and subjected to a second cold shock at an age of 15 months. For each individual fish, a breeding value was estimated (EBV_{15}) for stress-related cortisol using an animal model (described in chapter 4) with a fixed h^2 of 0.60. Although the EBV_{15}'s might be somewhat biased, because not all selection could be accounted for, they provide a more useful tool for further selection than the individual plasma cortisol concentrations, since

all available information was included (own cortisol response and cortisol response of, on average, 24 androgenetic sibs).

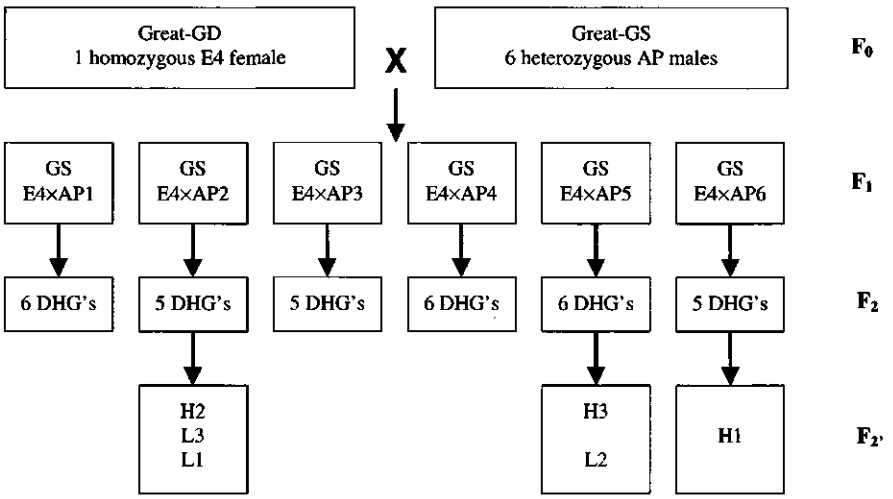

Figure 1: Schematic representation of pedigree from which the four homozygous sires and two homozygous dams were selected. The F_0 consisted of one homozygous domesticated E4 great-granddam (Great-GD) and six heterozygous wild AP great-grandsires (Great-GS). The F_1 consisted of six E4×AP full-sib families with five to 6 grandsires (GS) each. The F_2 consisted of 33 androgenetic double haploid progeny groups (DHG's) from which six were selected ($F_{2'}$) with either a high (H) or low (L) mean plasma cortisol during a cold shock at 4 months.

Table 1: Mean (±SD) plasma cortisol (nmol l^{-1}) at age 4 months and age 15 months of selected high (H) and low (L) F_2 androgenetic double haploid progeny groups (DHG's). Per DHG, the F_1 E4×AP full-sib family (FSF) the grandsire originated from, the number (n) of sires and dams per APG and the selected high or low sires (S) and dams (D) with their estimated breeding values for cortisol (EBV_{15}; nmol l^{-1}) are given.

F_2 DHG	FSF	Cortisol 4 months	Cortisol 15 months	n	Selected sires/dams (EBV)
H1	E4×AP6	927 ± 246^{a}	541 ± 171^{a}	22	D(369)
H2	E4×AP2	819 ± 350^{ab}	477 ± 135^{ab}	22	
H3	E4×AP5	789 ± 135^{ab}	541 ± 188^{a}	26	S(345)
					S(222)
					S(-69)
L3	E4×AP2	601 ± 141^{bc}	516 ± 157^{a}	35	
L2	E4×AP5	535 ± 218^{c}	508 ± 135^{ab}	24	S(-39)
					D(-60)
L1	E4×AP2	480 ± 190^{c}	372 ± 113^{b}	25	

2.2 Experiment 1 Homozygous isogenic strains

Four homozygous fertile F_2 sires with either a high cortisol EBV_{15} (S(345) and S(222)) or a low cortisol EBV_{15} (S(-69) and S(-39)) were selected for androgenetic reproduction (Table 1, Figure 2a). Androgenesis was carried out as described by Bongers et al. (1994). In brief, *c.* 1000 eggs per sire were UV irradiated, fertilised with milt and incubated at 24°C. Thirty minutes after fertilisation a 2 min heat shock was administered by transferring the eggs to a water bath of 40°C.

The first 21 dph, *c.* 150 larvae per sire were reared separately in 25 l aquaria at 25°C and fed freshly hatched *Artemia* nauplii at satiation three times a day. From 21 dph onwards, the four homozygous isogenic (HomIso) strains were housed in separate aquaria within an experimental unit connected to a recirculation system, consisting of a plate separator, a biological reactor and an UV treatment unit. Fish were fed a

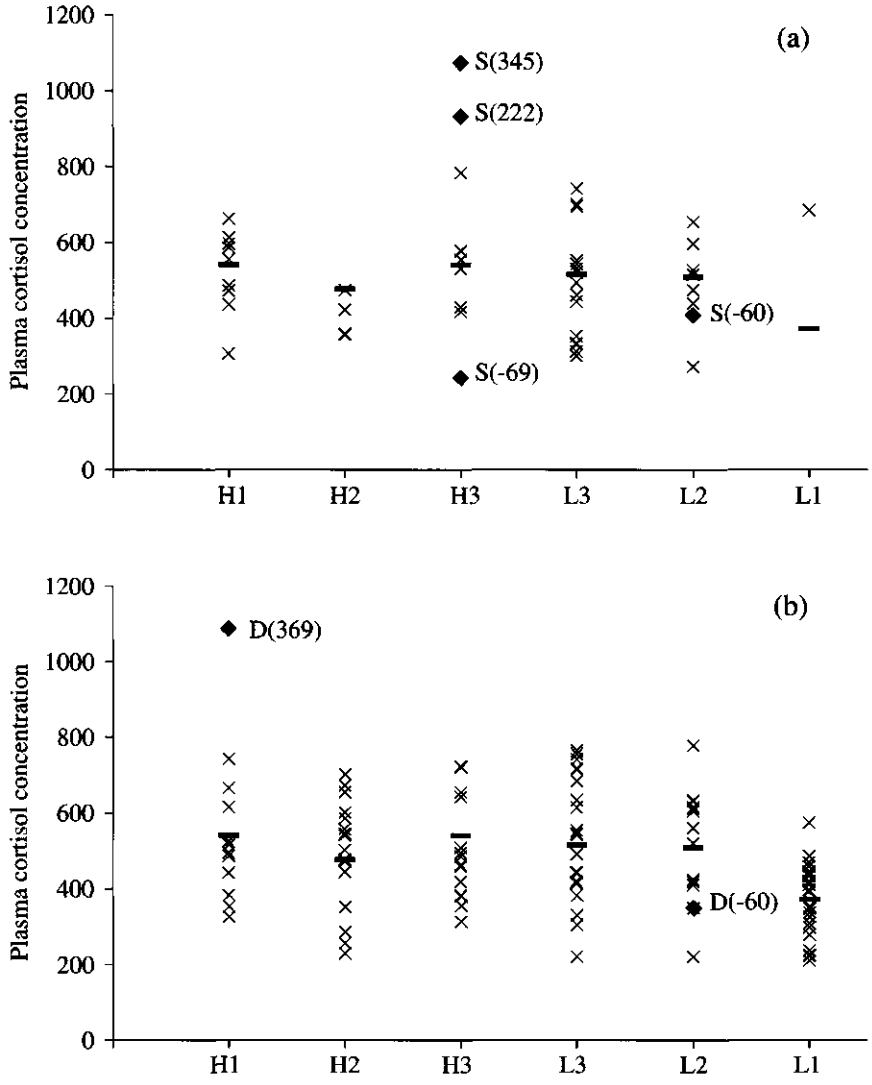

Figure 2: a) Plasma cortisol concentrations (nmol l^{-1}) of all sires (n = 47) present in the six selected DHG's (H1-3 and L1-3) at 20 min during a 9°C cold shock at an age of 15 months. b) Plasma cortisol concentrations (nmol l^{-1}) of all dams or sterile fish (n = 107) present in the six selected DHG's (H1-3 and L1-3) at 20 min during a 9°C cold shock at an age of 15 months. In both figures, diamonds represent the individuals selected for reproduction, crosses represent the unselected individuals and the bar represents the mean cortisol concentration of the group.

commercial trout pellet (Provimi, 91 series, Rotterdam, The Netherlands) at a daily ration of 20 g $kg^{-0.8}$. Food was administered for 8 h per day using conveyer belt feeders and daily increased assuming a feed conversion ratio of 1. Fish were weighed every 14 days and food rations were adjusted accordingly.

At 126 dph, each of the four HomIso strains was divided into four groups of 12 individuals. The 16 aquaria of the experimental unit were divided into four blocks of four aquaria (I: aqua 1 - 4, II: aqua 5-8, III: aqua 9-12 and IV: aqua 13-16). Within each block, one group of each HomIso strain was randomly allocated to one of the four aquaria (Proper and equireplicated block design). Based on a power analysis, the design of four aquaria per strain and 12 individuals per aquarium should enable us to significantly ($\alpha = 0.05$) detect a difference of *c.* 150 nmol l^{-1} between strains. Based on the EBV_{15}'s (Table 1) and a h^2 of 0.6, the probability that the actual difference in plasma cortisol concentrations at 20 min after a cold shock between the highest and the lowest strain would be 350 nmol l^{-1} was 90% with four strains (mean SD of the EBV_{15}'s was 90 nmol l^{-1}). Increasing the number of tanks per strain increases the power of detecting differences lower than 150 nmol l^{-1}, but such differences are too small to be biological significant in carp. Increasing the number of strains in the experiment would not improve the chance of finding larger differences, because the additional sires would have less extreme EBV_{15}'s than the sires already selected.

At 142 dph, all 16 aquaria were subjected to a 9°C cold shock and sampled 20 min after onset of the shock. A detailed description of the cold shock procedure is given in chapter 2. Aquarium 1 was sampled at 0920 hours (*c.* 1.5 h after feeding) and subsequent aquaria were sampled with 10 min intervals. This way, aquaria 1 - 16 were sampled within 2.5 h and the block design ensured that sampling intervals between groups of the same HomIso strain were similar between the strains. No groups with basal plasma cortisol levels were sampled at 142 dph. Instead, two aquaria with six individuals each were (again) sampled per strain at 165 dph without prior treatment to obtain an estimate of basal cortisol levels. In the period between 142 and 165 dph, treatment and rearing conditions were as before.

2.3 Experiment 2 Heterozygous isogenic strains

The same four homozygous sires (S(345), S(222), S(-69) and S(-39)) used in experiment 1 were crossed with two homozygous dams with either a high, D(369) or low, D(-60) cortisol EBV_{15} (Table 1, Figure 2b) to produce eight different heterozygous isogenic (HetIso) strains. After hatching, the HetIso strains were reared separately until

5 dph. At that day, ten groups were made with three individuals per HetIso strain in each group. The strains were reared communally until the day of sampling. Communal rearing was applied, because the 80 aquaria needed to test the eight HetIso strains separately (as in experiment 1) were not available. Furthermore, the sampling time becomes too large. At 21 dph, the ten groups were moved to a 140 l aquarium in the experimental unit. The rearing protocol of these groups was identical to that applied in experiment 1, except that these groups were not divided further at 126 dph.

At 142 dph, two groups were sampled at t = 0 min without prior treatment (controls). The remaining eight groups were subjected to the 9°C cold shock. Four groups were sampled at t = 20 min and four groups at t = 60 min after onset of the cold shock. This additional time point was included to obtain an indication of cortisol dynamics during the cold shock. Treatments and sampling times were randomly allocated to the ten groups. Group 1 was sampled at 0930 hours (1.5 h after feeding) and subsequent groups were sampled with 20 min intervals resulting in group 10 being sampled at 1230 hours. The respective sire and dam of an individual were identified afterwards using allele patterns at three polymorph carp microsatellite loci. These three microsatellites were selected based on genotype information of the sires and dams.

2.4 Sampling and plasma cortisol and DNA analyses

All fish in an aquarium were caught in one sweep with a tightly fitting net and immediately anaesthetised in water containing 0.3 g l^{-1} tricaine methane sulphonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) and 0.4 g l^{-1} $NaHCO_3$. After the fish were adequately anaesthetised (< 2 min), all fish were simultaneously removed from the anaesthetic and blood samples were taken by puncturing the caudal vessels using 1 ml syringes fitted with a 23 G needle rinsed with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany) as anti-coagulant. Blood samples were transferred to 2 ml tubes and immediately centrifuged for 5 min at 1500× ***g***. Plasma and blood cell pellets were stored separately at -20°C pending analyses. After blood sampling, individual body weights were scored. All fish in an aquarium were sampled within 10 minutes after capture in both experiments.

Plasma cortisol levels were determined by radioimmunoassay (RIA) (De Man et al., 1980). In other selective breeding programmes for stress, a parameter of the secondary stress response like plasma glucose is often incorporated as selection criterion (Fevolden et al., 1993; Pottinger and Carrick, 1999a). However, the cold shock stressor applied in the present experiments, does not induce apparent stress-related

changes in plasma glucose concentrations (see chapter 2). Therefore, analysis of plasma glucose was not included in the present experiments.

Genomic DNA, isolated from the blood cell pellets, was used to characterise the individual fish from experiment 2 with three microsatellites (MFW002, 040 and 046. Crooijmans et al., 1997 and chapter 5). The PCR was carried out as described by Crooijmans et al. (1996) and all three MFW's were combined in one multiplex set. This mixture was separated on a 6% denaturing polyacrylamide gel (Sequagel-6, National Diagnostics, Atlanta, GA, USA) using an ABI 373 automated sequencer (Perkin Elmer, Foster City, CA, USA). Gels were interpreted using Genescan and Genotyper software packages (Perkin Elmer).

2.5 Statistical analyses

2.5.1 Experiment 1 Homozygous isogenic strains

Per HomIso strain, aquarium means (12 individuals per aquarium) and standard deviations within an aquarium ($\hat{\sigma}_{within}$) and between replicate aquaria ($\hat{\sigma}_{between}$) were calculated for cortisol. Data were analysed for differences in plasma cortisol and $\hat{\sigma}_{within}$ between the four HomIso strains by ANOVA followed by Tukey's test for pairwise comparisons (Proc GLM, SAS Institute, 1990) using the following statistical model:

$$Y_{ij} = \mu + S_i + e_{ij} \qquad (1)$$

Where Y_{ij} is the observed body weight, cortisol concentration or $\hat{\sigma}_{within}$ for these traits (j = 1,..., 4), μ is the overall mean, S_i is the effect of the sire (i = S(345), S(222), S(-69), S(-39)) and e_{ij} is the residual error term. Residues from the model were normally distributed (Wilk-Shapiro test: W > 0.90) and variances were equal (Levene's test). P-values < 0.05 were regarded significant.

A breeding value (EBV_5) was estimated for each sire using the cortisol data of their androgenetic progenies and standard selection index theory:

$$\hat{EBV}_5 = \frac{2nh^2}{1+2nh^2} \times (\bar{x} - \mu) \qquad (2)$$

Where n is the number of progeny per sire (n = 48), h^2 is the heritability (h^2 = 0.60 for cortisol in common carp: chapter 4), x is the mean value of the progeny group and μ is the population mean. Data were analysed for a possible correlation between EBV_{15} and EBV_5 of the sires. In the text, correlation is expressed as Pearson correlation

coefficients (r). To verify whether the responses at 5 and 15 months were genetically the same trait, this r was tested against the expected $r = 1$ using the 95% confidence interval (95% c.i.) of r (Neter et al., 1996). A realised heritability was calculated for upward and downward selection using h^2 = R/S (Falconer and Mackay, 1996), where R is the selection response (difference between mean phenotypic value of progeny of the selected parents and the mean of the whole parental generation before selection) and S the selection differential (mean phenotypic value of the individuals selected as parents expressed as a deviation from the whole population mean before selection). The upward and downward realised heritabilities were averaged to one mean realised heritability.

2.5.2 *Experiment 2 Heterozygous isogenic strains*

For cortisol at t = 20 min, the $\hat{\sigma}_{within}$ and $\hat{\sigma}_{between}$ was calculated per strain. Cortisol data from experiment 2 were analysed per sample point (t = 0, 20 or 60) for dam, sire and interaction effects using the statistical model:

$$Y_{ijkl} = \mu + Aq_i + D_j + S_k + DS_{jk} + e_{ijkl} \qquad (3)$$

Where Y_{ijkl} is the observed plasma cortisol concentration (l = 1, 2, 3), μ is the mean at that time point, Aq_i is the effect of the aquarium (i = 1, 2 or 1,...,4), D_j is the main effect of the dam (j = D(369), D(-60)), S_k is the main effect of the sire (k = S(345), S(222), S(-69), S(-39)), DS_{jk} the interaction between dam_j and $sire_k$ effects and e_{ijkl} the residual error term. Based on least square means from model 3, the general combining abilities (GCA) of the sires and dams for cortisol at the different sample points were calculated. GCA's were calculated as parental least square mean minus the overall mean. Data were analysed for possible correlations between the EBV_{15} of the sires and dams and the GCA's at t = 20 and t = 60 min. Similar to experiment 1, the r's of the correlations between the EBV_{15} and the two GCA's were compared with the expected value of 1 using the confidence intervals of r.

Per dam or sire, cortisol data were analysed for differences between the three time points by ANOVA followed by Tukey's test for pairwise comparisons using the model:

$$Y_{ijk} = \mu + Aq_i + T_j + e_{ijk} \qquad (4)$$

Where Y_{ijk} is the observed plasma cortisol concentration (k_{dam} = 1,..., 104; k_{sire} = 1,..., *c.* 52), μ is the mean per dam or sire, Aq_i is the effect of the aquarium (i = 1, 2 or 1,...,4), T_j is the sample point (j = 0, 20, 60) and e_{ijk} the residual error term. Residues from the

models 3 and 4 were normally distributed (Wilk-Shapiro test: W > 0.90) and variances were equal (Levene's test). P-values < 0.05 were regarded significant.

3 Results

3.1 Experiment 1 Homozygous isogenic strains

At 142 dph, the mean individual weight (± SD) of the fish was 54.3 ± 19.5 g. Stress-related plasma cortisol and basal plasma cortisol concentrations of the four HomIso strains are shown in Table 2. The ranking in the stress-related cortisol levels (S(345) > S(222) > S(-39) > S(-69)) is equal to the ranking that would be expected based on the EBV_{15}'s. Strain S(345) had significantly higher cortisol levels than the strains S(-39) and S(-69). Strain S(222) was only significantly higher than strain S(-69). The difference in plasma cortisol levels between the highest and the lowest responding HomIso strain was 354 nmol l^{-1}.

Table 2: Mean (±SD) stress-related plasma cortisol (nmol l^{-1}) at 142 dph (*c.* 5 months) and basal plasma cortisol levels (nmol l^{-1}) at 165 dph of the four homozygous isogenic strains. Letters denote significant ($P < 0.05$) differences between strains.

Strain	Cortisol	Basal cortisol
S(345)	776 ± 115^{c}	84 ± 6^{b}
S(222)	635 ± 89^{bc}	31 ± 3^{a}
S(-39)	505 ± 100^{ab}	47 ± 4^{a}
S(-69)	422 ± 58^{a}	79 ± 5^{b}

The basal levels measured at 165 dph are significantly higher in both the S(-69) and S(345) compared to the basal levels found in S(-39) and S(222). The EBV_{15} based on the performances of the sires were positively correlated with the EBV_5 estimated based on the performance of the androgenetic progenies ($r = 0.97$). The 95% c.i. (0.14 - 1) did not include zero, but did include the expected r value of 1. The mean realised heritability was 0.28 (h^2-upward: 0.36; h^2-downward: 0.20). The mean (± SD) $\hat{\sigma}_{within}$ of the four strains for cortisol was 150 ± 37 nmol l^{-1} and no significant

differences in $\hat{\sigma}_{within}$ were found between the HomIso strains. The mean (±SD) $\hat{\sigma}_{between}$ for cortisol was 91 ± 24 nmol l^{-1}.

3.2 Experiment 2 Heterozygous isogenic strains

At 142 dph, the number of fish in the ten groups varied between 18 and 24 (mean ± SD: 21 ± 1.6) due to some mortalities in period between 5 and 21 dph and the mean individual weight was 106.8 ± 34.0 g. The plasma cortisol levels measured in the eight HetIso strains at t = 0, 20 and 60 min after onset of the cold shock are shown in Figure 3. No significant differences were found between the HetIso strains in the observed levels at t = 0 (basal levels; least square mean: 48 nmol l^{-1}) and at t = 60 min. At t = 20, the D(-60)×S(222) strain showed a significant lower response compared to five other HetIso strains.

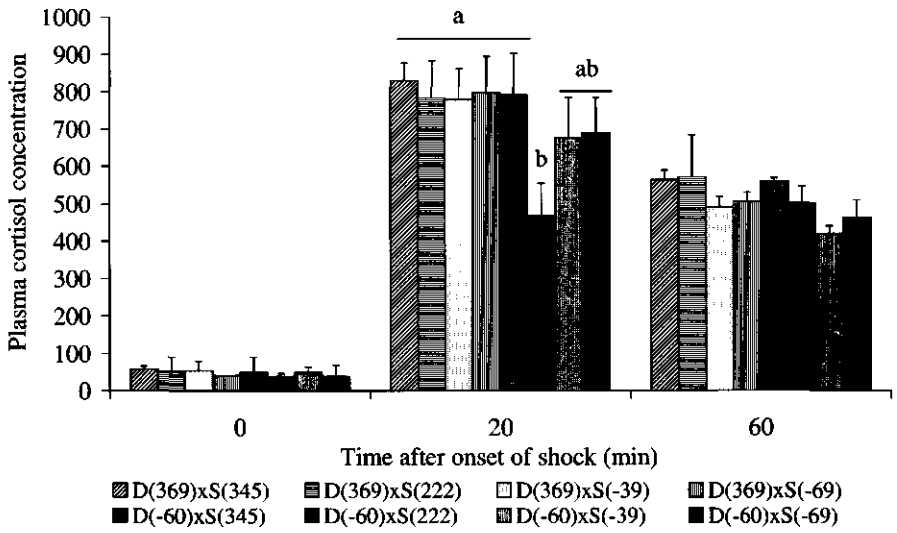

Figure 3: Mean (± SE) plasma cortisol levels (nmol l^{-1}) in eight heterozygous isogenic strains measured at t = 0, 20 and 60 min after onset of a 9°C cold shock. S = sire; D = dam. Values between brackets represent the EBV_{15} of that individual. Letters denote significant differences in plasma cortisol levels at t = 20 min.

Significant dam and sire influences on the cortisol levels were found at t = 20 min and a significant sire effect at t = 60 min (Table 3). At none of the three sample points, a significant dam × sire interaction was found. Progenies from D(369) had higher plasma cortisol levels at t = 20 and 60 min, but only significantly different at t = 20 min. At 20 min, progenies from S(345) had significantly higher plasma cortisol levels (807 ± 194 nmol l^{-1}) compared with levels found in progenies from S(222) (619 ± 243 nmol l^{-1}). At 60 min, progenies from S(345) had significantly higher levels (560 ± 74 nmol l^{-1}) than progenies from S(-39) (450 ± 76 nmol l^{-1}). The Pearson correlation coefficients for EBV_{15} and the GCA at t = 20 was 0.51 (95% c.i. -0.36 - 1). The *r* for the EBV_{15} and the GCA at t = 60 min was 0.88 (95% c.i. 0.01 – 1). Both 95% c.i. are large, but include the expected *r*-value of 1. Based on model 4, progenies from all sires and dams showed a significant increase in plasma cortisol at 20 and 60 min after onset of the cold shock. Except for progenies from S(222), progenies from the other three sires and the two dams all showed a significantly lower plasma cortisol at t = 60 min compared to t = 20 min after onset of the shock. The mean (±SD) $\hat{\sigma}_{within}$ and $\hat{\sigma}_{between}$ for plasma cortisol levels at t = 20 min were 141 ± 51 and 180 ± 40 nmol l^{-1}, respectively.

Table 3: *P*-values for dam, sire and interaction (Dam × Sire) effects on plasma cortisol levels of eight heterozygous isogenic strains at different sample points.

Sample point	Dam	Sire	Dam × Sire
t = 0 min	0.13	0.91	0.88
t = 20 min	0.000	0.02	0.24
t = 60 min	0.14	0.02	0.82

4 Discussion

The aim of the present experiments was to develop homozygous and heterozygous isogenic strains with divergent stress-related cortisol responses. Androgenesis was chosen to obtain DH individuals, because of the presence of both dams and sires in the DH progeny groups, enabling us to produce heterozygous isogenic strains from these F_2 parents without sex-reversal. Fertile males (31%) were easily identified by checking for sperm appearance after application of gentle pressure on the abdomen. The remaining 69% of the fish in the selected progeny groups were either female or sterile (Based on section of some of the remaining 27 progeny groups, *c.* 48% of the individuals is expected to be female). The percentage of sterile fish in androgenetic progenies can be considerable (13 – 94%) as shown by Bongers et al. (1999). A further problem was presented by a low ovulation response of a relative high percentage of the androgenetic DH female carp, similar to the lower responses found in gynogenetic DH female carp (Komen et al., 1992). These problems resulted in a restricted availability of possible DH parents. As Figure 2b illustrates, especially the selection of a dam with a low cortisol response (and EBV_{15}) was hampered by sterility and ovulation problems. Whether this sterility or lower ovulation response is related to the cortisol response or caused otherwise is not clear.

The estimated breeding values (EBV_{15}) for the DH sires and dams in the six selected DHG's were based on the stress response during a 9°C cold shock observed at an age of 15 months. The mean cortisol levels in the selected H1-3 are much lower than those found at 4 months (Table 1; chapter 4). As a result, differences in mean plasma cortisol between the selected groups have become smaller. Genetically, the stress response at 15 months might be regarded as a different trait compared to the stress response at 4 or 5 months and EBV's based on the response at 15 months might not be predictive for the response at another age. To investigate whether the stress responses at different ages are genetically different traits, EBV_5, based on the HomIso strain performance (experiment 1), and GCA's, based on the HetIso strain performances (experiment 2), were compared with the previously calculated EBV_{15}'s. Although the correlation coefficients are only based on four to six observations and estimated inaccurately given the broad 95% c.i., none of them provides evidence that the stress-related cortisol release at 5 and 15 months are different traits.

In experiment 1, the maximal difference in plasma cortisol concentrations at t = 20 min was found between the expected highest S(345) and lowest S(-69) HomIso

strain. The magnitude of the difference (354 nmol l^{-1}) corresponded with the expected difference based on the EBV_{15}'s. This, together with the equal ranking in cortisol concentrations in the HomIso strains and the EBV_{15} of the sires, indicates that additive genetic effects have a prominent effect on the stress-related cortisol response due to a cold shock. This observation is confirmed by the significant sire and dam effects found in experiment 2. Although the largest difference in plasma cortisol levels in this experiment was also c. 350 nmol l^{-1} (found between the D(369)×S(345) and the D(-60)×S(222) strain), differences between the strains were smaller than expected based on examination of values for observed and expected ($\cong 0.5\times(EBV_{15,dam} + EBV_{15,sire})$) performances of the HetIso strains. A possible explanation could be the influence of non-additive genetic effects, but based on the results from experiment 2 ($P_{D\times S} = 0.14$) such effects did not play a role. Practical circumstances forced us to use a communal rearing design for experiment 2. An advantage of communal rearing is the opportunity to adjust the data for possible aquarium effects. However, mixing genotypes (≅HomIso and HetIso strains) might result in competition (social interaction) between genotypes, possibly resulting in different cortisol responses compared with those found in a separate rearing design. Although influences of social hierarchy on the stress response have been reported in a number of fish species (Noakes and Leatherland, 1977; Ejike and Schreck, 1980; Peters et al., 1988; Pottinger and Pickering, 1992), no reports dealing with social hierarchy in common carp have been published so far. To resolve the effect of possible interactions between genotypes, the stress response of a particular genotype should be tested simultaneously in a number of different 'fish environments', which is possible using different strains of genetically identical individuals.

The maximum differences found in both experiments are comparable to the difference of 268 nmol l^{-1} found by Pottinger and Carrick (1999b) and lower than the mean difference of *c.* 550 nmol l^{-1} found by Fevolden et al. (1999). Both compared crosses of consistently high responding mature rainbow trout (*Oncorhynchus mykiss* Walbaum) with crosses of consistently low responding trout after one generation of selection. However, these crosses can be used for further selection rounds, whereas the strains used in the present study are the end product of the selection programme, due to the homozygous state of the sires and dams used.

Next to testing the inheritance of the stress response, experiment 1 offered us the possibility to examine the phenotypic variance (V_P). Because variances due to genetic factors (V_A, V_D and V_I) are absent in homozygous isogenic strains, the mean $\hat{\sigma}_{within}$ for cortisol of the four HomIso strains (150 ± 37 nmol l^{-1}) gives a estimate of the

phenotypic variation due to environmental effects only ($V_P = V_E$). Given the high h^2 of 0.6 for stress-related cortisol in carp (chapter 4), this phenotypic variation (V_P) should theoretically be lower than that within an androgenetic DHG ($V_P = V_A + V_E$) or outbred strain ($V_P = V_A + V_D + V_I + V_E$). However, the mean $\hat{\sigma}_{within}$ of the HomIso strains is similar to the mean $\hat{\sigma}_{within}$ of 33 androgenetic DHG's (187 ± 54 nmol l^{-1}; experiment described in chapter 4) or within two outbred strains (172 ± 42 and 156 ± 29 nmol l^{-1}, respectively; Tanck, unpublished results). Strictly, this would indicate a small contribution of V_A compared to the V_E, which is in contradiction with results from the present experiments and the high h^2 reported previously in chapter 4. However, given the differences in environmental circumstances between the experiments and the difficulty of accurately estimating variances with the relative low number of fish used in the different experiments (115 – 660), drawing conclusions based on these $\hat{\sigma}_{within}$'s is hazardous. The mean $\hat{\sigma}_{between}$ in experiment 1 (91 ± 24 nmol l^{-1}) is similar to the $\hat{\sigma}_{between}$ found with outbred carp (112 ± 17 nmol l^{-1}; Tanck, unpublished results), indicating that aquarium and sampling effects do not have a different effect on cortisol values in isogenic strains compared with outbred strains.

Contemporary experiments with other F_1 hybrid strains (see chapter 7) have shown that next to the height of the peak, the total amount of cortisol measured during a cold shock can be consistently different between strains (prolonged response). Because the sires and dams used in the present experiments were only selected for their response at 20 min, an additional aim of experiment 2 was to investigate the stress response at an additional point (t = 60 min). The results indicate that all sires and dam had similar cortisol dynamics (except for the height of the levels). Although most other selective breeding programmes for stress also use cortisol responses at a fixed time point as selection criterion (Fevolden et al., 1993, 1999; Pottinger and Carrick, 1999), the total amount of cortisol measured during a stress response might become a more important selection criterion than just the height of the (expected) peak in future selection programmes. Furthermore, the selected DH sires and dams used as parents in the present experiments have been selected based on their stress response due to a cold shock. The first step for further research would be to subject progenies from these sires and dams to e.g. confinement or handling stress and examine the results for possible genotype × stressor interactions.

In conclusion, studying the stress response in homozygous- and heterozygous isogenic strains confirmed the role of additive genetic effects in the height of the stress-related cortisol response. Although only a limited number of crosses were tested in

experiment 2, the results did not reveal significant non-additive genetic effects for this trait. It can be argued, therefore, that the best method to change the stress responsiveness in common carp would be through selective breeding rather than through crossbreeding.

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Chapter 7

Effect of genotype on stress response dynamics in common carp (*Cyprinus carpio* L.)

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To be submitted

Abstract

The aim of the present study was to investigate the plasma cortisol, glucose and lactate dynamics during a cold shock in different genotypes and at different ages. Four homozygous parents were crossed in a 2×2 design, resulting in four F_1 hybrid strains, which were subjected to a 9°C cold shock and sampled at either 5 or 7 months. The experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. Significant dam and sire effects on the total amount of cortisol measured during the cold shock were found, but no significant dam × sire interaction effect. Although no significant difference was found between the cortisol response dynamics at 5 or 7 months, the results indicate that further research into that field is justified. The increase in plasma glucose during the cold shock observed in the genotype with the prolonged cortisol response could be caused by the glucocorticoid action of cortisol, but the observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related. Age did not have any apparent influence on plasma glucose and lactate dynamics in both control and shocked fish.

1 Introduction

In an earlier study, a test was developed to quantify the stress response of common carp (*Cyprinus carpio* L.) using a 3 hour cold shock (ΔT 9°C) as stressor (Chapter 2). Results presented in chapters 4 and 6 showed that genetic variation exists in stress-related plasma cortisol levels at 20 and 60 min after onset of a cold shock. However, 'complete' response patterns during the cold shock have, so far, only been studied in one male isogenic strain, thereby limiting the observations to a single genotype. In this genotype, the cold shock induced a rapid rise in plasma cortisol with a peak level occurring within 20 min after onset of the shock. Between 20 and 180 min cortisol values were decreasing but remained significantly higher compared to the basal cortisol levels measured in the matching controls. Previous results have also indicated that stress responses can be influenced by the age or developmental stage of the fish (Larsen, 1976; Pottinger et al., 1995; Chapter 2). Therefore, the aim of the present study was to study the plasma cortisol, glucose and lactate dynamics during a cold shock of different genotypes and at different ages.

To achieve this aim, four homozygous parents were crossed in a 2×2 design, resulting in four F_1 hybrid strains. These fish were sampled at either 5 or 7 months (age effect). These ages were chosen, because male carp become mature at *c.* 6 months of age under the rearing conditions in our facilities (Bongers et al., 1997) and sexual

maturation has proven to influence the (height of the) stress response in a number of (salmonid) fish species (Sumpter et al., 1987; Pottinger et al., 1995). To avoid possible differences in stress response due differences in weight rather than age, the fish sampled at either 5 or 7 months were fed with different feeding levels resulting in similar weights at the day of sampling (Experiment 1). The higher feeding level was similar to the level used in the study described in chapter 2, but the lower feeding level had not been used before. As a possible interaction of (low) feeding level with age could not be excluded, the experiment was repeated, but this time all fish were fed the lower feeding level.

2 Materials and methods

2.1 Broodstock

Two homozygous dams (E4 and R3R8-69-45) were crossed with two homozygous males (R3R8 (yy) and E4AP (yy)), to obtain four F_1 hybrid strains (E×R, E×EA, R×R and R×EA). The E×R strain is the strain that was also used in a previous study (Chapter 2). The R3R8-69-45 dam is a high antibody producing animal originating from a divergent selection programme for antibody production (Wiegertjes et al., 1995). The E4AP (yy) sire originates from an androgenetic progeny group with an on average high cortisol level 20 min after onset of the cold shock (chapter 4).

2.2 Rearing of F1 hybrid strains

The first 21 days post hatching (dph), the rearing protocols were similar in both experiments. In each experiment, *c.* 300 larvae per strain were reared separately in 25 l aquaria at 25°C and fed freshly hatched *Artemia* nauplii at satiation three times a day. From 21 dph onwards, each of the four strains was divided into two groups of *c.* 100 fish each (Figure 1). These groups were housed in separate aquaria within an experimental unit connected to a recirculation system, consisting of a plate separator, a biological reactor and an UV treatment unit, and fed a commercial trout pellet (Provimi, 91 series, Rotterdam, The Netherlands). In experiment 1, group 1 sampled at 142 dph (*c.* 5 months) was fed a ration of 17 g $kg^{-0.8}$ d^{-1}. Group 2, sampled at 213 dph (*c.* 7 months), was fed a ration of 11 g $kg^{-0.8}$ d^{-1}. In experiment 2, both groups received the same feed ration of 11 g $kg^{-0.8}$ d^{-1} from 21 dph onwards. In both experiments, food was administered for 8 h per day using conveyer belt feeders. The amount of feed was

increased daily assuming a feed conversion ratio of 1. Fish were weighed every 14 days and feeding schedules adjusted accordingly. On the sampling days, fish that were sampled received 50% of the feed ration for that day 1 hour before the onset of the cold shock.

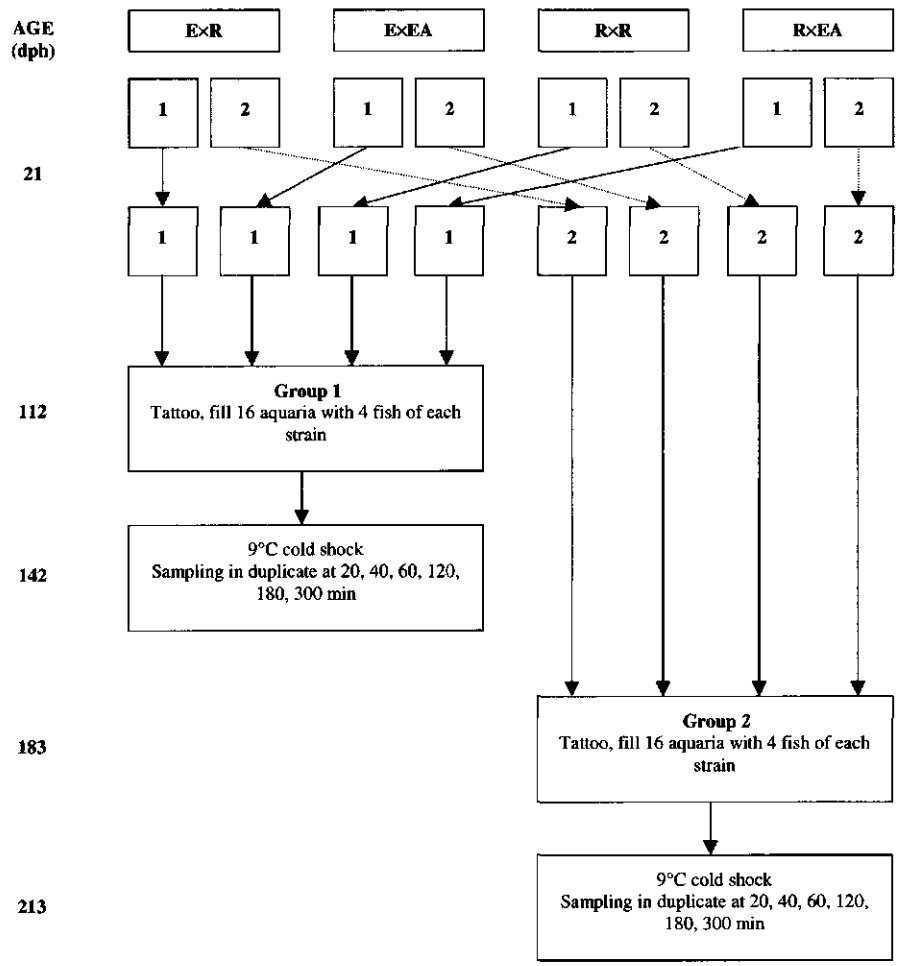

Figure 1: Schematic representation of the experimental design used in experiment 1. At the top, the four genotypes are shown, each divided in two groups (1 & 2; 100 individuals each) at 21 days post hatching (dph). Group 1 was fed 17 g $kg^{-0.8}$ d^{-1} and sampled at 142 dph and group 2 was fed 11 g $kg^{-0.8}$ d^{-1} and sampled at 213 dph. The same design was also used in experiment 2, but in that experiment both groups were fed 11 g $kg^{-0.8}$ d^{-1}.

One month prior to the sampling day, all fish in a group destined for sampling were marked with a small tattoo to distinguish the strains (Figure 1). Thereafter, each aquarium in an experimental unit consisting of two rows of eight 140 l aquaria was stocked with four randomly chosen individuals per strain (communal rearing). At the sampling days, fish in six randomly chosen aquaria in the upper row and fish in six randomly chosen aquaria in the lower row were subjected to a 9°C cold shock (described in detail in Chapter 2). Sampling points were chosen at t = 20, 40, 60, 120, 180 and 300 min after onset of the shock. Each sampling point thus consisted of a randomly chosen aquarium from the upper row and a randomly chosen aquarium in the lower row. Because the two shocked aquaria per sample point were sampled subsequently, the cold shocks in the lower row were initiated 10 min after the cold shocks in the upper row. Four control sample points were chosen at t = 0, 80, 140 (Exp. 2) or 200 (Exp. 1), and 320 min after onset of the shock using one of the four remaining non-shocked aquaria.

2.3 Sampling and plasma analysis

All 16 fish in an aquarium were caught by one sweep of a tightly fitting net and anaesthetised immediately in water containing 0.3 g l^{-1} tricaine methane sulphonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) and 0.4 g l^{-1} $NaHCO_3$ (pH *c.* 7.5). Blood samples were taken by puncture of the caudal vessels using 1 ml syringes fitted with a 23 G needle and rinsed with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany) as anti-coagulant. After blood sampling, the individual body weights (BW) and the tattoo of the fish were scored. All fish in an aquarium were sampled within 10 min after capture. Blood was transferred to Eppendorf vials and centrifuged immediately for 10 min at 1500× ***g*** and 4°C. Plasma was stored at -20°C pending analyses. Per sampling day, fish in three randomly picked aquaria were killed and dissected to obtain testis weights (TW), which were used to calculate the testis-somatic-index (TSI; TW / BW ×100%) for each individual.

Plasma samples were analysed for cortisol, glucose and lactate concentrations. Cortisol levels were determined by radioimmunoassay (RIA) (De Man et al., 1980). Plasma glucose was analysed with a commercial kit (no. 124 028, Boehringer Mannheim GmbH, Mannheim, Germany) using a 96 well microtitre plate. The final solution was measured at 420 nm. Lactate concentrations were analysed using a lactate kit (no. 735-10, Sigma Aldrich, St. Louis, MO, USA) adapted to be used with microtitre plates (measurement wavelength: 540 nm).

2.4 Statistical analyses

Next to possible effects of experiment, age, treatment and genotype on the mean plasma cortisol, glucose and lactate concentrations, we also investigated if plasma cortisol, glucose and lactate concentrations per treatment changed within time (t, 0 – 180 min) and if changes in time within treatments differed between the two ages or the four genotypes. This was done by analysing whether regression coefficients were significantly different from zero and/or different between treatments, ages or genotypes. The complete model used to analyse the plasma cortisol, glucose and lactate data between 0 and 180 min was (Proc GLM, SAS 6.12; SAS Institute, 1990):

$$Y_{ijklmn} = \mu_{..} + EA_{ij} + ET_{ik} + EG_{il} + TG_{kl} + R(EAT)_{ijkm} + b_k t_{ijklmn} + b_{jk} t_{ijklmn} + b_{kl} t_{ijklm} + e_{ijklmn} \quad (1)$$

where Y_{ijklmn} = the observed cortisol plasma cortisol, glucose or lactate concentration (n = 4), EA_{ij} = the interaction effect of experiment and age (i = 1, 2; j = 142, 213 dph), ET_{ik} = the interaction effect of experiment and treatment (k = control, shock), EG_{il} = the interaction effect between experiment and genotype (l = E4×R3R8 (yy), E4×E4AP (yy), R3R8-69-45×R3R8 (yy), R3R8-69-45×E4AP (yy)), TG_{kl} = the interaction effect of treatment and genotype, $R(EAT)_{ijkm}$ = the effect of replicate aquaria (m = 1, 2) nested within the interaction of experiment, age and treatment, b_k = the regression coefficient for treatment on time (t_{ijklm}), b_{jk} the regression coefficient for the age × treatment interaction, b_{kl} = the regression coefficient for the treatment × genotype interaction and e_{ijklmn} = the residual error term. Experiment and age did not have a significant influence on the recorded parameters and were left out of the model. Other possible interactions had no significant influences on the recorded parameters and were also left out of model 1. Genotype was not included as fixed effect, because we were only interested in difference between genotypes within a treatment. For the regression coefficients b_k, b_{jk} and b_{kl}, 95% confidence intervals (95% c.i.) were estimated to enable comparison of the different b's.

Per genotype and sampling day, the mean cortisol values in the shocked aquaria from t = 20 – 180 min and the basal level recorded at t = 0 min were used to calculate the area under the curve (AUC) between t = 0 and 180 min in the shocked aquaria. This AUC is an estimation of the total amount of plasma cortisol measured during the cold shock. AUC data were analysed for experiment, age and genotype effects experiment using the model:

$$Y_{ijkl} = \mu + E_i + A_j + G_k + e_{ijkl} \quad (2)$$

where Y_{ijkl} = the observed AUC (l = 1) of a genotype (k = E×R, E×EA, R×R, R×EA) at a certain age in an experiment. Model 2 with inclusion of a replicate component (R(EA)) was used to analyse the body weight and TSI data for age and genotype effects. No significant interaction effects were found.

AUC data were also analysed for possible dam, sire and interaction effects using the model:

$$Y_{ijklm} = \mu + E_i + A_j + D_k + S_l + DS_{kl} + e_{ijklm} \quad (3)$$

where Y_{ijklm} = the observed AUC (m = 1,..., 4), μ = the overall mean, E_i = the fixed effect of experiment (i = 1, 2), A_j = the fixed effect of age (j = 142, 213 dph), D_k = the effect of the dam (k = E4, R3R8-69-45), S_l = the effect of the sire (l =R3R8 (yy), E4AP (yy)), DS_{kl} = the effect of the dam × sire interaction and e_{ijklm} = the residual error term. The residues from the different models were normally distributed (Wilk-Shapiro test, W > 0.90) and variances were equally distributed within the variable classes (Levene's test) making data transformations unnecessary. When necessary, a Bon-Ferroni correction was applied to correct the α for the number of comparisons made.

3 Results

The mean individual body weights and TSI at the sampling days in both experiments for the different genotypes are shown in Table 1. Significant differences in mean body weight were found between the genotypes at the end of the communal rearing period. The (least square) means based on all four sampling days were: R×EA, 68.7 g; R×R, 67.0 g > E×R, 62.3 g > E×EA, 56,8 g. The (least square) mean TSI at 142 dph (1.98%) was significantly lower (P_A < 0.05, Table 2) than the mean TSI at 213 dph (3.44%). TSI for the E×R and the E×EA genotypes (4.21 and 3.39%) were higher than the TSI of the R×R and R×EA genotypes (1.67 and 1.57%).

Table 1: Feed ration (day 21 – sampling day), mean (±SD) individual body weight and mean (±SD) testis-somatic-index (TSI) of the different genotypes (n=64) sampled at 142 or 213 days post hatching (dph) in experiment 1 or 2.

	Experiment 1		Experiment 2	
	142 dph	213 dph	142 dph	213 dph
Feed (g $kg^{-0.8}$ d^{-1})	17	11	11	11
Genotype	Body weight (g)			
E×R	71.6 ± 11.7	71.8 ± 10.5	30.3 ± 4.4	75.5 ± 11.2
E×EA	64.2 ± 20.1	64.8 ± 13.5	27.8 ± 5.8	70.8 ± 14.1
R×R	79.1 ± 24.7	80.5 ± 25.9	28.4 ± 5.9	80.2 ± 17.3
R×EA	80.5 ± 17.5	78.5 ± 13.8	34.1 ± 6.6	82.0 ± 15.0
Genotype	TSI (%)			
E×R	4.1 ± 2.3	5.0 ± 3.1	1.7 ± 1.0	6.1 ± 1.6
E×EA	2.4 ± 1.7	4.0 ± 2.7	2.8 ± 1.2	4.2 ± 2.4
R×R	2.3 ± 1.7	3.2 ± 1.7	0.2 ± 0.1	1.0 ± 0.9
R×EA	1.7 ± 0.9	1.8 ± 0.5	0.6 ± 0.4	2.3 ± 1.6

Table 2: *P*-values for experiment × age (EA) and treatment × genotype (TG) interaction effects on plasma cortisol, glucose and lactate levels and *P*-values for the regression of these parameters on time for treatments (b_k), the age × treatment interaction (b_{jk}) and the treatment × genotype interaction (b_{kl}) (model 1). *P*-values are also given for age (A) and genotype (G) effects on the total amount of cortisol measured during the cold shock (AUC; model 2) and the testis-somatic-index (TSI; model 2').

Trait	P_{EA}	P_{TG}	P_{b_k}	$P_{b_{jk}}$	$P_{b_{kl}}$
Cortisol	0.000	0.000	0.000	0.195	0.000
Glucose	0.000	0.000	0.000	0.073	0.000
Lactate	0.000	0.158	0.378	0.005	0.386
Trait	P_A	P_G			
AUC	0.212	0.003			
TSI	0.000	0.000			

The (least square) mean plasma cortisol concentrations at the different sample days are shown in Table 3. Age did not have a significant influence on the mean cortisol level measured at the different time points, but a significant experiment × age interaction was found (P_{EA}, Table 2). The plasma cortisol levels in shocked fish decreased faster at 142 dph (b_{jk} ± SE: -0.92 ± 0.21 nmol.l^{-1} min^{-1}) compared to the decrease observed at 213 dph (b_{jk} ± SE: -0.054 ± 0.021 nmol.l^{-1} min^{-1}), but this difference was not significant (Table 2). In control fish, no differences were found in the mean plasma cortisol between the genotypes. Significant differences were found between the genotypes (P_{TG}) in the shocked fish (R×R > R×EA, E×R > E×EA). In three of the four genotypes, cortisol levels decreased significantly between 20 and 180 min after onset of the shock (b_{kl}, Table 4; Figure 2), but no significant decrease in time was found in the R×R genotype. A significantly higher AUC was found in the R×R genotype compared to those of the E×R and E×EA genotypes. Significant dam (P_D = 0.002) and sire (P_S = 0.010) effects were found for the AUC, but no significant interaction effects (P_{DS} = 0.221). In all four genotypes, the plasma cortisol levels in shocked fish returned to basal levels (< 50 nmol l^{-1}) when the temperature was back at 25°C (t = 300 min).

Table 3: Least square means values (based on model 1) for plasma cortisol, glucose and lactate concentrations observed at the two sampling days in both experiments. Letters denote significant ($P < 0.05$) differences between the sample days.

Experiment	Age	Cortisol (nmol l^{-1})	Glucose (mmol l^{-1})	Lactate (mmol l^{-1})
1	142	219^{b}	9.07^{a}	2.91^{a}
1	213	289^{a}	6.21^{b}	2.16^{c}
2	142	271^{a}	4.34^{c}	2.70^{b}
2	213	256^{ab}	4.61^{c}	2.66^{b}

Table 4: Regression coefficients (b_{kl}) and 95% confidence intervals (95% c.i.) of the regression of cortisol and glucose concentrations during a cold shock (t = 20 – 180 min) for treatment × genotype interaction (model 1).

Genotype	Cortisol		Glucose	
	b_{kl}	95% c.i.	b_{kl}	95% c.i.
E×R	-1.01	-1.43 – -0.59	-0.010	-0.016 – -0.004
E×EA	-0.87	-1.29 – -0.45	-0.016	-0.022 – -0.010
R×R	0.15	-0.27 – 0.57	0.011	0.005 – 0.017
R×EA	-1.22	-1.64 – -0.80	-0.009	-0.015 – -0.003

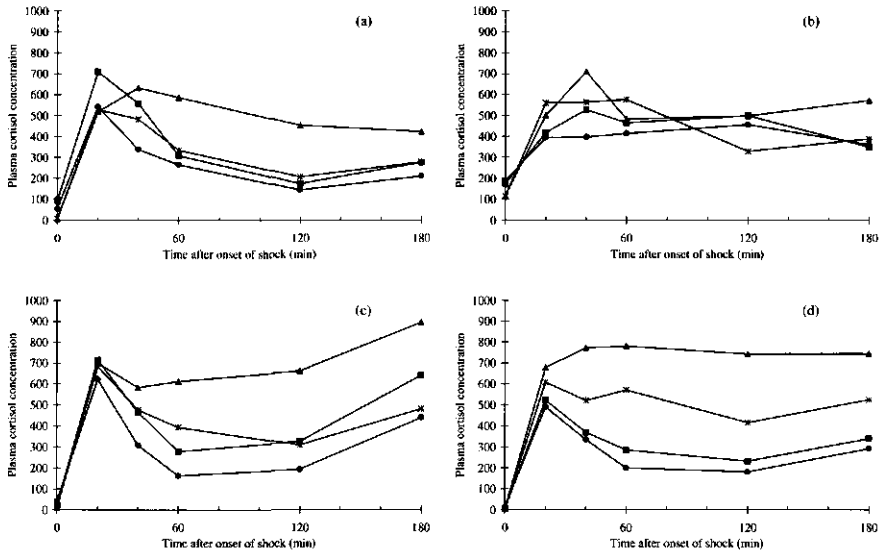

Figure 2: Plasma cortisol concentrations (nmol l^{-1}) during a 9°C cold shock in the E×R (■), the E×EA (●), the R×R (▲) and the R×EA (*) genotypes at an age of 142 (a) and 213 dph (b) in experiment 1 and at 142 (c) and 213 dph (d) in experiment 2.

The (least squared) mean plasma glucose levels at 142 and 213 dph in experiment 1 and 2, shown in Table 3, resulted in a significant experiment × age interaction (P_{EA}; Table 2). At both ages, plasma glucose levels changed similarly

(P-value b_{jk}; Table 2). In control fish, plasma glucose levels decreased significantly faster between t = 0 and 180 min (b_k ± SE: -0.018 ± 0.001 mmol l^{-1} min^{-1}) than in shocked fish (b_k ± SE: -0.006 ± 0.002 mmol l^{-1} min^{-1}). In control fish, glucose concentrations in the E×EA genotype decreased significantly slower (b_{kl}: -0.011 mmol l^{-1} min^{-1}) compared to fish of the other three genotypes (b_{kl}: -0.018, -0.021 and -0.022 mmol l^{-1} min^{-1}, for the E×R, R×R and R×EA genotype, respectively). The mean glucose concentration in control fish of the R×EA genotype (6.13 mmol l^{-1}) was significantly higher than the concentration in control fish of the E×EA genotype (4.45 mmol l^{-1}). In shocked fish, plasma glucose levels increased significantly in fish of the R×R genotype, whereas glucose levels decreased significantly in the other three genotypes (b_{kl}, Table 4; Figure 3a). Significant influences of genotype on mean plasma glucose levels in the shocked groups were also observed (R×R, 8.53 mmol l^{-1} > R×EA, 7.41 mmol l^{-1} > E×R, 6.51 mmol l^{-1} > E×EA, 4.42 mmol l^{-1}).

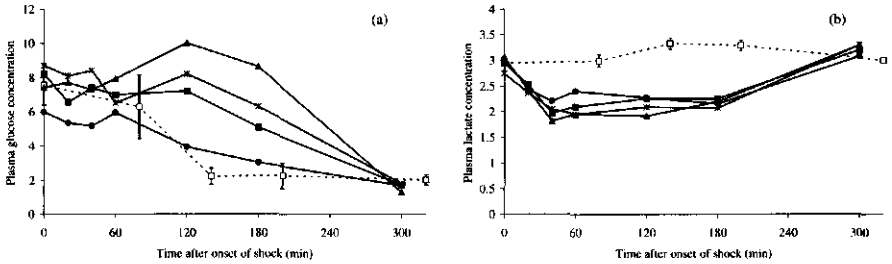

Figure 3: Plasma glucose (a) and lactate (b) concentrations (mmol l^{-1}) during a 9°C cold shock in the E×R (■), the E×EA (●), the R×R (▲) and the R×EA (*) genotypes based on least square means estimated using model 1 and data from all four sampling days within the experiments. Control levels (❒) are presented as averaged least square mean of all four genotypes (± SD).

The (least square) mean plasma lactate concentrations at the different sample days are shown in Table 3. These resulted in a significant experiment × age interaction (Table 2). At 213 dph, the lactate levels decreased significantly between 20 and 180 min in the shocked fish (b_{jk} ± SE: -0.0014 ± 0.0005 mmol l^{-1} min^{-1}), but no significant decrease was found in the shocked fish at 142 dph (b_{jk} ± SE: 0.0007 ± 0.0005 mmol l^{-1} min^{-1}) during the same time period. In control fish, no differences were found between the genotypes, but the mean lactate level in the shocked fish of the E×EA

genotype (2.27 mmol l^{-1}) was significantly higher than that in fish of the R×R genotype (2.05 mmol l^{-1}; Figure 3b). Two hours after the cold shock (t = 300 min), plasma glucose and lactate levels in shocked fish were similar to those observed in control fish sampled at t = 320 min.

4 Discussion

For cortisol, no difference in the height of the levels was found between the age-groups. Although the fish sampled at 213 dph had a higher TSI, which indicates an increased maturation compared to 142 dph (Degani et al., 1998), the difference in development might not be sufficient to produce a noticeable difference. This is supported by the conclusion that the difference in TSI between the ages is probably mainly caused by the lower TSI at 142 dph in experiment 2 (Table 1). This lower TSI is most likely caused by the lower weights at that sampling day (Degani et al., 1998). Next to the height of the response, no significant effect of age on the response pattern could be found. However, in shocked E×R, E×EA and R×EA fish, plasma cortisol levels at 213 dph in experiment 1 (Figure 2b) decreased significantly slower (data not shown) than the observed decreases at the other three sampling days, including the 'replicate sampling' at 213 dph in experiment 2 (Figure 2d). The reason for the difference between the samplings at 213 dph is unclear. Although fish in the two experiments were produced in different years, rearing circumstances were kept as equal as possible to avoid differences in rearing history between experiments. Seasonal influences on the stress response, as reported by Pottinger & Carrick (2000), are unlikely in our case, since water temperatures and light regimes are kept constant throughout the year in our facilities. In addition, both samplings at 213 dph took place in June. Although the observations at 213 dph in experiment 1 might be due to a day effect, they would justify further experiments to examine whether age or sexual maturation has an effect on the stress response pattern in common carp.

The cold shock induced significant cortisol stress responses in all four genotypes at each of the different sampling days. Similar to previous experiments (Chapter 2), the E×R genotype generally showed a peak level at 20 min followed by a significant decrease ($b_{kl} < 0$). This response pattern was also observed in the E×EA and R×EA genotypes. The R×R genotype, however, consistently showed a different stress response with a peak level at 40 min and no significant decrease ($b_{kl} > 0$) in cortisol levels at the subsequent sample points during the cold shock (Figure 2, Table 4). This prolonged response resulted in a significantly higher total amount of circulating cortisol measured

during the cold shock (AUC) in this genotype compared to the E×R and E×EA genotypes. Since circulating cortisol levels reflect the balance between release and clearance rate, this higher cortisol AUC in the R×R genotype can be due to a prolonged release e.g. due to different perceptions of the stress or differences in feedback mechanisms, or a slower clearance rate (cortisol-cortisone shuttle) or combination of both. Pottinger and Moran (1993) found a difference in cortisol dynamics in two strains of rainbow trout (*Oncorhynchus mykiss*) during confinement stress, which were most likely related to differences in the cortisol – cortisone shuttle, as shown by changes in the simultaneously measured plasma cortisone levels. Under increasing or normal temperature circumstances (t = 180 – 300 min), cortisol is cleared from the circulation in R×R fish, since levels in shocked fish at 300 min were similar to levels measured in the controls at 320 min. This indicates, that the R×R genotype has a functional cortisol metabolism at those temperatures. Therefore, differential interactions of lower water temperatures with cortisol release and metabolism mechanisms between the genotypes could also be responsible for the difference in observed stress response patterns.

In a communal rearing design, social interactions between genotypes can influence the height of basal cortisol levels within a genotype (Ejike and Schreck, 1980; Winberg and Lepage, 1998). Furthermore, effects of social interaction on the stress-related cortisol response pattern have also been reported (Øverli et al., 1999). Although no significant differences in basal levels were found in the present study and the response pattern of the standard genotype E×R is similar to that observed previously in a separate rearing design (Chapter 2), it can not be excluded that the observed response patterns of the different genotypes are only valid under the present rearing conditions. Before the R×R genotype is used in other experiments, this genotype should also be tested in a separate rearing design.

In the present experiments, fish were fed 1 hour before start of the experiments, which is in contrast with the majority of the previous experiments described in Chapter 2. Comparison of the results from both studies revealed that feeding the fish prior to a cold shock had no apparent effect on plasma cortisol and lactate patterns. As could be anticipated, feeding had a significant influence on the plasma glucose levels and patterns in the sampled fish. In the control fish of the present experiments, initial plasma glucose concentrations varied between 4 and 12 mmol l^{-1} (depending on experiment, age and genotype) and decreased within three hours after feeding (t = 140 min) to basal levels similar as observed in earlier experiments (*c.* 2.0 mmol l^{-1}; Chapter 2). The variation in initial glucose levels between experiments and ages (P_{EA}, Table 2) was

caused by the higher feeding ration of 17 g $kg^{-0.8}$ d^{-1} for the group sampled at 142 dph in experiment 1. The differences in initial glucose levels between genotypes, which were responsible for differences in the b_{kl}'s for glucose in the control fish and differences in mean plasma glucose between genotypes, might be truly genetic differences (e.g. differences in digestion and resorption capacity). However, these differences might also be due to the communal rearing design applied in the present experiments. In such a design, differences in competitive ability between the genotypes within an aquarium can result in unequal feed intakes between the genotypes (McCarthy et al., 1992; Kadri et al., 1996; Ryer & Olla, 1996), resulting in related unequal initial plasma glucose concentrations. These, in turn, probably resulted in different growth rates between the genotypes, since the rank order in initial plasma glucose levels corresponds with the rank order in mean body weight at the different sampling days (Table 1).

A significant decrease in plasma glucose levels was found in both control and shocked fish, but the mean decrease between 0 and 180 min (b_k) was significantly slower in shocked fish compared with control fish. Because a cold shock in non-fed shocked E×R fish did not induce an increase in plasma glucose levels, the slower decrease observed in this genotype in the present experiments was probably due to a slower glucose metabolism, most likely caused by the lower water temperature itself, rather than through an increased glucose release in the shocked fish. The effect of lower temperatures is mediated through a lower activity of enzymes involved in glucose transport and glycogenesis in combination with temperature-related changes in cell membranes (Dey & Farkas, 1992; Williams & Hazel, 1994), which have to be passed. Possibly similar mechanisms were responsible for the positive relationship between glucose uptake and water temperature observed in fish intestines (Groot et al., 1983; Houpe et al., 1996). Next to shocked E×R fish, plasma glucose concentrations also decreased at similar rates in shocked E×EA and R×EA fish (Figure 3a; Table 4). In the R×R genotype, however, shocked fish showed a significant increase, compared to the initial levels, from 40 min onwards. Because of this relatively slow glucose response, this increase could be due to the glucocorticoid function of cortisol (Van der Boon et al., 1991). Since this increase was absent in the genotypes with a lower cortisol AUC, it could be argued that increased cortisol levels during a prolonged time period (> 60 min) are necessary to obtain a noticeable plasma glucose increase within the sampling period. However, E×R, E×EA and R×EA fish also showed a prolonged cortisol response at 213 dph in experiment 1 (Figure 2b), but didn't show an increase glucose levels after 60 min. This indicates that if the increase in plasma glucose observed in the R×R genotype is cortisol-related, it appears to be genotype specific.

Similar to glucose, the experiment × age effect on the height of the lactate levels is most likely due to the higher feeding level of fish sampled at 142 in experiment 1. Furthermore, the differences found between the ages are relatively small and similar to variations in the daily basal levels (Tanck, unpublished results). Because the decrease in plasma lactate levels in shocked fish mainly occurred within 20 min after onset of the cold shock and remained stable during the rest of the shock, no significant difference between the b_k of control and shock fish was found (*P*-value $b_k = 0.365$). Between the genotypes, no differences in plasma lactate dynamics were found. Similar to glucose, the differences between genotypes in mean lactate levels during the cold shock are caused by different initial lactate levels.

Genetic variation in plasma cortisol responses due to different stressors have been reported for a number of (mainly salmonid) fish species (see e.g. review by Afonso et al., 1998) and additive genetic effects on the stress-related cortisol response have also been found in common carp (Chapters 4 and 6). However, most selection experiments, including ours (described in Chapter 6), use cortisol levels (sometimes in combination with other parameters) measured at a single point during or after application of the stressor. Based on these single point observations, no conclusions can be drawn whether a strain or genotype is a high / low or quick / slow responder or about the duration of the response. In the present study, the four genotypes did not have significantly different cortisol levels at t = 20 min after onset of the cold shock (= selection criterion used in our selection experiment), but only differed in the levels observed at the later sample points and in the total amount of cortisol measured during the cold shock. Since, for instance, a possible cortisol-related glucose response could only be observed in the R×R genotype with the prolonged response, such a genotype would be a valuable addition for a panel of genotypes (Bongers et al., 1998) to be used in different experiments aimed at, for example, finding the mechanisms responsible for the different stress responses and the consequences of these responses on other physiological processes. In a practical selection programme for stress responsiveness, selection against genotypes with a prolonged cortisol response could be important. Taking this into consideration, a selection criterion using information on cortisol levels at several time points could be advantageous. Although a selective breeding programme could incorporate a step in which androgenetic or gynogenetic homozygous individuals are made, using isogenic strains to obtain a response pattern for a single genotype to be used as selection criterion is too labour-intensive and expensive to be considered as a feasible practical approach. Multiple sampling of the same individual might already be more practical, but since cannulation is also unsuitable from a practical point of view, the multiple samples have

to be taken with reasonably large intervals to prevent possible influences of the previous sampling and habituation. However, similar to Pottinger and Moran (1993), stress response patterns can be obtained for a particular strain or family by sampling related individuals at the different time points. This way, the stress response patterns could become a valuable selection criterion in those selective breeding programmes for stress responsiveness, which are based on family selection.

The present experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. Significant dam and sire effects on the total amount of cortisol measured during the cold shock were found, but no significant dam × sire interaction effect. Although no significant difference was found between the cortisol response dynamics at 5 or 7 months, the results justify further research into that field. The increase in plasma glucose during the cold shock observed in the genotype with the prolonged cortisol response could be caused by the glucocorticoid action of cortisol, but the observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related. Age did not have any apparent influence on plasma glucose and lactate dynamics in both control and shocked fish.

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Chapter 8

General discussion

This thesis describes a selection experiment aimed at producing isogenic carp strains (*Cyprinus carpio* L.) with either a high or low stress-related cortisol response. Isogenic strains of fish are commonly used in research (Bongers et al., 1998), but using doubled haploid individuals and isogenic strains to estimate genetic parameters as part of a selection experiment is no common practice. In the first paragraph, the results obtained in the present project are compared with the original objectives and the efficacy of a cold shock as a stressor is discussed. In paragraph 2, some of the problems related to large-scale selection programmes for stress response in fish and the potential contributions of the developed strains to solve these problems are discussed. The (dis)advantages of using androgenetic progenies in a breeding programme are discussed in paragraph 3.

1 Exploring the genetic background of stress response in common carp

As the results of the different experiments show, the cold shock proved to be capable of inducing repeatable cortisol responses in common carp. These responses were of similar magnitude as seen with other commonly used acute stressors like confinement, netting, handling and line-and-rod catching (Yin et al., 1995; Weyts et al., 1997; Pottinger, 1998). However, unlike these stressors, which usually also invoke a secondary stress response (e.g. rapid increase in plasma glucose or lactate; McDonald and Robinson, 1993; Van Raaij et al., 1996; Pottinger, 1998), cold shocks did not induce an (expected) increase in either plasma glucose or lactate concentrations, at least not within five hours after onset of the shock (see chapter 2 and 7).

A stress-related rapid increase in plasma glucose concentration is mainly caused by the glycogenolytic action of catecholamines (CA; Wendelaar Bonga, 1997). CAs are generally released under conditions that require enhanced blood oxygen transport and the mobilisation of energy substrates, e.g. during hypoxia (Claireaux and Dutil, 1992; Perry and Reid, 1994; Van Raaij et al., 1996) or strenuous activity (e.g. struggling, forced swimming; Nielsen et al., 1994; Pottinger, 1998). Both did not occur during the cold shock and it can be hypothesised that the absence of the rapid hyperglycaemia could be explained by a lack of a primary CA response during the cold shock. As a second hypothesis, the lack of a secondary response might be due to a temperature related suppression on the actions of the released CAs or on the glucose and lactate metabolisms (Pastoureaud, 1991; Houpe et al., 1996). However, both hypotheses can not be substantiated without actual measurements of CA levels during a cold shock.

A third explanation might be found in the fact that common carp experience rapid temperature changes on a more or less daily basis, both under natural and aquacultural circumstances. In shallow lakes or ponds, for instance, differences between day and night temperatures can be considerable, depending on the depth of the water body (Milstein and Svirsky, 1996; Rothuis et al., 1998). Excessive rainfall, e.g. during the tropical rainy season, can also result in sudden temperature drops (Reading and Clarke, 1999). In larger water bodies, carp can be subjected to rapid temperature changes due to the presence of both horizontal and vertical temperature gradients (Augustyn and Szumiec, 1985; Díaz et al., 1998), while moving from the water surface to the bottom and vice versa during feeding, for instance. It can be argued that displaying a primary and, especially, a secondary stress response at each rapid temperature change within the optimal temperature range would become detrimental for carp. Therefore, natural selection might have acted against displaying a secondary response against this form of temperature stress.

The question whether the lack of a secondary response is due to a temperature-related suppression of the secondary response could be investigated by subjecting carp reared at a lower temperature to a rapid temperature increase (16 → 25°C, for instance). An absence of the secondary response under those circumstances would indicate that sudden temperature changes are most likely perceived as no- or mild stressors by the carp. Furthermore, it would be interesting to investigate whether a cortisol response also occurs during a rapid temperature decrease under natural circumstances.

Due to the fact that cold shocks did not induce a noticeable stress-related change in either plasma glucose or lactate concentrations, our selection experiment was based on the cortisol response only. The heritability estimated in chapter 4 clearly indicated that additive genetic effects play an important role in the stress-related cortisol response and that the magnitude of the stress response is a heritable trait in common carp. The importance of additive genetic effects for stress response in carp was further demonstrated in chapters 6 and 7, and no noticeable non-additive genetic effects were found. Subsequent family and individual selection within the androgenetic E4×AP progeny groups, used to estimate the heritabilities, resulted in isogenic strains with either a relatively high or low cortisol response at 20 min after onset of the shock (Chapter 6). In an additional parallel experiment, the stress response patterns of four other, readily available, isogenic strains (Chapter 7) were examined. Three of these strains (E×R, E×EA and R×EA) showed a peak response at 20 min followed by a rapid decrease in cortisol levels. This pattern is similar to the patterns found in the isogenic strains from the selection experiment (Chapter 6). The remaining R×R strain showed a

similar plasma cortisol level at 20 min, but this level did not significantly decrease during the cold shock. Interestingly, the R×R strain was the only strain tested that showed an increase in plasma glucose during the cold shock. Based on their stress response pattern, the different isogenic strains from the two different experiments (selection and 'parallel') can be divided in at least two types. Type I shows a relative short cortisol response with either a low or high peak level at 20 min (e.g. standard strain E×R and the strains from the selection experiment), whereas Type II shows a prolonged cortisol response (R×R strain). Additional types, like a slow responder, could also exist and would be a welcome addition to the types already available. Therefore, the stress response patterns of other genotypes should also be studied.

In conclusion, the two major aims of the project have been fulfilled. First, it has been demonstrated that the magnitude of the cortisol stress response is a heritable trait in common carp. Second, the different experiments using isogenic strains demonstrated the existence of different stress-related cortisol response patterns. As our carp strains are genetically uniform, these strains offer the possibility to separately investigate the effects of genotype, environmental circumstances and stress response on a number of important traits and, thus, form a valuable toolbox for further research. However, due to the limited number of genotypes presently available, generalisation of the obtained results should be undertaken with care as the biological significance of the differences in plasma cortisol concentrations and cortisol dynamics in these isogenic strains remains to be investigated.

2 Selective breeding for stress response in fish

Research has shown that stress-related cortisol response is a hereditary trait in a number of important aquacultural fish species, including Atlantic salmon, *Salmo salar*, rainbow trout, *Oncorhynchus mykiss* (Fevolden et al., 1991, 1993b; Pottinger and Carrick, 1999b) and common carp (this thesis). The stress-related cortisol response has been incorporated as a trait in experimental selection programmes, but not yet in large-scale commercial selection programmes. This is mainly due to the fact that a number of important questions related to selective breeding for this trait are still unclear. These questions are: 1) in which direction should the selection for cortisol stress response be performed: although selective breeding for stress-related cortisol response is mainly considered as an indirect selection method to improve disease resistance, the relationship between cortisol and disease resistance appears to be ambiguous. 2) what kind of stressor and which type of response should be used as selection criterion in a

selective breeding programme, and 3) how important is the testing environment (separate or communal testing design) for the stress response of a genotype?

2.1 Direction of selection

Similar to terrestrial animals, general disease resistance is an important trait in fish. Therefore, improvement of disease resistance through direct or indirect selection is an important issue. As deliberate exposure of the breeding population to diseases should be prevented, parameters that are genetically correlated to disease resistance and which can be measured in live and healthy fish (= indirect selection) are preferred (Fevolden et al., 1991). Obviously, immunological parameters like e.g. antibody production (Wiegertjes et al., 1995) and lysozyme concentration (Fevolden et al., 1999) are candidate traits. Furthermore, due to the intimate relationship between stress response and disease resistance (Snieszko, 1974), the stress response (and particular the stress-related cortisol response) is also regarded as a potentially valuable tool in the selection process for increased disease resistance. However, the interactions between cortisol and the immune system are complex, leading to an ambiguous relationship between cortisol and disease resistance.

Similar to mammals, the immune system in fish can be divided into innate (a-specific) immune responses and acquired (specific) immune responses. The latter can be further divided into the humoral (B-cell mediated) and the cellular (T-cell mediated) response. Differential interactions between cortisol and the different parts of the immune system exist. For instance, Weyts et al. (1998b) found that especially activated B-lymphocytes of carp are sensitive for cortisol (*in vitro*) leading to programmed cell death (= apoptosis) of these cells. On the other hand, cells of the innate immune system appeared to be less sensitive to cortisol and neutrophilic granulocytes were even rescued by cortisol from apoptosis *in vitro* (Weyts et al., 1998a). This implies that selection for e.g. a high stress-related cortisol response could lead to an improved innate immune response under stressful conditions, but a decreased humoral immune response. Depending on the immune response(s) needed to fight off a specific pathogen, this would then result in an improved or decreased disease resistance. For example, Gross and Colmano (1971) showed that chickens selected for a low stress-related plasma corticosterone level were more resistant to Marek's disease virus and *Mycoplasma gallisepticum* infection, and more susceptible to *Escherichia coli* infection when compared to the high line. The important role of corticosterone in these results was demonstrated by the fact that chemicals, which block the corticosterone synthesis, caused an increased resistance to viral infections and a decreased resistance to *E. coli*

infection (Colmano and Gross, 1971; Gross and Siegel, 1973). Similarly, Fevolden et al. (1992, 1993a) found that the disease resistance in Atlantic salmon and rainbow trout could be influenced by selection for stress-related cortisol, but contrasting results were obtained when different bacterial pathogens (*Aeromonas salmonicida*, *Vibrio anguillarum* and *Renibacterium salmoninarum*) were used.

The strains with divergent cortisol responses developed in the current project and isogenic strains from a divergent selection programme for antibody response to a synthetic antigen (DNP-KLH; Wiegertjes et al., 1995) will be used to further study the relationship between disease resistance and cortisol response (NWO-ALW programme: The interaction between immune competence and stress responses in relation to fish health problems; 1997-2001). Until more is known about this relationship, indirect selection based on immunological parameters or direct selection for general disease resistance might be more successful. The latter is e.g. practised by the Norwegian Salmon Breeding Company, who decided to select for increased survival after challenge test with furunculosis (*A. salmonicida*; bacterial disease) and infectious salmonid anaemia (ISA; viral disease). These diseases were chosen, because resistance against furunculosis or ISA only show a weak (negative) genetic correlation. Therefore, selection of candidates superior for both diseases should ascertain that selection is not too disease specific (Gjøen et al., 1997).

2.2 Selection criterion

Chronic vs. acute?

Fish in an aquacultural environment encounter acute stressors like netting and confinement, for instance, during weighing and sorting. This will result in a primary and secondary stress response, but the effects of this acute stressor on growth, reproduction and/or the immune system (= tertiary stress response) will probably be minimal. On the other hand, a chronic stressor like crowding, adverse water conditions or social interactions will result in a prolonged stress response, which has severe effects on e.g. growth, reproduction and the immune system (maladaptation). Most selection experiments for stress response, including ours, use the response to an acute stressor as selection criterion. It can be argued, however, that using the response to a chronic stressor as selection criterion could yield better results in preventing maladaptation, especially if the responses to acute and chronic stressors are genetically different traits. Appropriate traits to measure the response to a chronic stressor are not available yet. Instead, traits to measure the response to an acute stressor are used. During the initial

phase of stress, fish generally show an increase in plasma cortisol and glucose concentrations. However, during prolonged application of a stressor, adaptation processes at the level of sensory input, appraisal of the stimuli and/or execution of the responses, may cause primary and secondary stress responses to attenuate (see e.g. Pickering and Stewart, 1984; Tort et al., 1996, Chapter 2), thus making these traits useless to measure the response on the longer term.

One 'universal' stress response?

It can be questioned, whether fish display a 'universal' stress response in reaction to different stressors or that each single (or group of) stressor(s) induce(s) a specific response. So far, no experiments have been published that studied the stress response of fish to another stressor than the one they were selected for. A preliminary experiment indicated that the lines selected for their cortisol response to a cold shock responded in a similar fashion to another acute stressor (N. Ruane, personal communication), but genotype × stressor interactions could occur. Now that isogenic strains with different cortisol stress response types are available, these genotypes should, therefore, be subjected to different acute and chronic stressors and possible genetic correlations should be estimated. This way, the validity of the approach to use the response to an acute stressor as selection criterion in a selection programme to prevent tertiary responses due to many different stressors could be tested.

Cortisol and/or catecholamines?

Pottinger and Carrick (1999a) showed that selection of individual rainbow trout based on their high or low cortisol response to confinement, did not result in correlated responses for glucose response and vice versa. If the rapid mobilisation of glucose during stress is regulated through the release of catecholamines, then it is possible that the two major neuro-endocrine pathways involved in the stress response, the hypothalamus – sympathetic nerves – chromaffin cell (HSC) axis and the hypothalamic – pituitary – interrenal cell (HPI) axis, are not closely coupled in this salmonid species. Therefore, a selection programme based on the cortico-steroidogenic response alone might fail to account for other elements of the stress response (Pottinger and Carrick, 1999a).

Peak value or response pattern?

Most selection programmes for stress use parameter values measured at a single time point. In our programme, this was at 20 min after onset of a 9°C cold shock. As discussed in chapter 7, cortisol dynamics during a cold shock can vary between

genotypes and a selection criterion based on measurements at multiple time points during (and after) application of the stressor could be more valuable than a selection criterion based on a single observation during the stressor.

2.3 Testing environment

Social interactions in salmonids are known to influence individual feed consumption (Metcalfe, 1986; Kadri et al., 1996) and dominance feeding hierarchies have been reported in many salmonids including rainbow trout (McCarthy et al., 1992), Arctic charr (*Salvelinus alpinus* L.; Olsen and Ringø, 1999) and Atlantic salmon (Metcalfe et al., 1989). In these species, effects of rank within a dominance hierarchy on basal and stress-related cortisol levels have been studied extensively (e.g. Noakes and Leatherland, 1977; Ejike and Schreck, 1980; Pottinger and Pickering, 1992; Øverli et al., 1999; Sloman et al., 2000). In common carp, social interactions start to appear at the end of the first month of life (length = 15 – 30 mm) and the interaction between juveniles is based on the principle of schooling relationships without dominance and on mutual attraction by individuals and imitative contacts (Panyushkin, 1989). Although Panyushkin (1989) did not observe aggressive contacts, effects of social interaction on feed related parameters like growth have been described in carp. For instance, the so-called 'shoot carp' (= strong positive skew in the frequency distribution of length) occur when food is scarce and no larger fish are present in a group. Fast growing fish will then dominate and slow or even suppress the growth of other fish in the group (Wohlfarth, 1977; Vilizzi and Walker, 1999). Similarly, differences in competitive ability caused an increased difference in absolute growth rates between carp strains under communal rearing compared to the differences when the strains were reared in separate ponds. In both testing environments, however, the rank order in growth rates between the strains was similar (Wohlfarth and Moav, 1985).

So far, no reports describing the possible effect of social interaction on the stress response of carp have been published. However, some results described in the present thesis (chapters 4 and 6) suggest that a stress response of a particular genotype might be different under variable 'fish environments' and that these responses might be regarded as genetically different traits. For instance, in an (unpublished) experiment to study the repeatability of the stress response in carp, two outbred strains were subjected to three consecutive cold shocks with a month interval and individual stress responses were monitored and used to estimate repeatabilities. Between the samplings, the fish within a strain were mixed and redistributed over the available aquaria. For each individual fish, this procedure resulted in different 'fish environments' at each of the sampling days.

The estimated low repeatabilities are in apparent contrast with the high estimated heritability for stress related cortisol as reported in Chapter 4. The low repeatability estimate could be explained if genotype × 'fish environment' interactions played an important role and the three consecutive stress responses should, therefore, be regarded as genetically different traits. Testing stress responses of different strains in a communal rearing design, as described in chapters 6 and 7, could also produce different results compared to separate testing designs due to possible effects of interactions between the strains on the responses. To study the possible effects of the presence of other genotypes on the stress response, the isogenic carp strains should, therefore, be tested under separate and communal rearing circumstances (Similar to the design used by Wohlfarth and Moav (1985) to test difference in growth rates). The obtained data can then reveal whether there is a difference in the stress responses of the genotypes between the rearing designs, and if so, whether the responses are correlated.

3 Androgenetic progenies in selection experiments

This section focuses on some of the pros and cons of including (an) androgenetic reproduction step(s) in a selection experiment or breeding programme, although most of the comments also apply for gynogenetic reproduction. The main reasons for choosing androgenesis instead of gynogenesis in the present thesis were the shorter generation intervals of males compared to females and the presence of both males (yy) and females in the androgenetic progeny groups, which makes subsequent production of isogenic strains much easier. As shown, androgenetic (and gynogenetic) progeny groups can be used to estimate heritabilities based on principles described by Bijma et al. (1997) and Bongers et al. (1997a). The estimates based on such a design are expected to be more accurate compared to e.g. a full-sib family design when the estimated heritability (h^2) is lower than 0.35. Above this value, there is even a clear disadvantage in using doubled haploid (DH) groups (Bijma et al., 1997). Therefore, if the expected size of the h^2 to be estimated is unknown, it would be wise to use a more conventional design using either full- or half-sib families, particularly because of the extra labour and time needed for production and subsequent rearing of the desired number of DH groups compared to conventional families. However, next to the expected low h^2 for stress-related cortisol based on the repeatabilities and h^2 estimations for stress response in Atlantic salmon and rainbow trout (Fevolden et al., 1993b; chapter 4), an important reason for us to estimate heritabilities for stress-related traits using the DH design was that it could be easily incorporated within our selection experiment aimed at creating isogenic strains with either a high or low stress response using androgenesis. The first androgenetic

generation consists of a desired number of DH progeny groups from which homozygous sires are chosen for a second androgenetic reproduction resulting in homozygous isogenic strains. To create heterozygous isogenic strains (= F1 hybrids), both homozygous sires and dams can be picked from the DH group(s). If sires and dams are selected randomly just to create isogenic strains without any particular trait, there is no need to measure the trait in the individual fish within the DH group(s). However, if the aim is to create isogenic strains selected for a particular trait like antibody response (Wiegertjes et al., 1995), maturation (Bongers et al., 1997b) or stress, the sires and dams within the DH progeny group have to be measured for this trait. Furthermore, a number of DH groups would have to be made to increase the genetic variation present within the population from which the parents will be selected. Based on data from these DH progeny groups, a heritability can be estimated for the trait under selection (Bongers et al., 1997a). Although the confidence interval of a h^2 might be rather large, depending on the number of progeny groups and the height of the estimated h^2, this estimate can be very valuable in determining whether there is any additive genetic variation present for the trait in the population and if so, whether the required level can be achieved in only one round of selection. If the goal is to create isogenic strains in only two generations, only two rounds of selection can be performed ($F_0 \rightarrow F_1$ and $F_1 \rightarrow F_2$). Although results from the present thesis show, that one round ($F_1 \rightarrow F_2$) can be enough to create strains with significant divergence for the selected trait (see chapter 6), further selection rounds might be necessary to reach a required level for the trait. Homozygous animals with the desired characteristics can be crossed to create a new heterozygous starting population for selection. An advantage of this approach is that by including a homozygous generation within a selection programme, the homozygous generation (and subsequent generations if no new heterozygous fish are introduced from outside the population) will be free of any detrimental recessive alleles. Similarly to estimating heritabilities for a trait or clearing detrimental recessive alleles from a whole population, androgenetic DH progenies of a male candidate genitor can be used to determine the breeding value and the genetic load of a single sire by measuring the performance of the androgenetic progeny or expression of deleterious genes in its androgenetic progeny, respectively (Van der Lende et al., 1998; Tave, 1999).

Once individuals in DH groups have matured, homozygous and heterozygous isogenic strains can be produced. As shown in chapter 6, these strains can be used for dissection of the phenotypic variance in variances due to genetic and environmental factors. Further uses of isogenic strains in research are numerous. Within a larger selection programme they could be used e.g. to estimate genetic correlations between

traits measured in different environments (G×E interactions). Since estimates of these G×E interactions can vary considerable in fish (Gjedrem, 1992), creating a number of isogenic lines to examine the presence and magnitude of such interactions can be very relevant within a larger programme and even determine whether different lines should be developed for different environments.

Theoretically, androgenetic progenies are 100% homozygous. However, as results presented in chapter 5 show, residual heterozygosity can occur in androgenetic DH individuals. Based on these results, additional controls have been included in our androgenesis procedure. Previously, two or more batches of eggs were irradiated, fertilised, but not heat shocked at the end of each session. These haploid batches served as UV irradiation controls, since incomplete irradiation would lead to normal diploid larvae, which can be easily morphologically distinguished from haploid larvae (Bongers et al. 1994). However, these controls give some general information about the UV irradiation, but not about a specific egg batch irradiated during the session. In the new procedure, a sample (± 100 eggs) of each batch of irradiated eggs is incubated separately to serve as UV irradiation controls, thereby providing information about the specific egg batch. If the irradiation in a specific egg batch was insufficient, the matching UV control will demonstrate that and the larvae from this batch can be discarded. Although the percentage of (partly) heterozygous individuals might be low, it must be taken into account since e.g. the DH design used to estimate heritabilities assumes total homozygosity of the DH progenies and presumed isogenic strains might not be genetically pure.

However, the major obstacle for including a complete androgenetically produced homozygous generation in a selection programme might be found in the difficulty to find fertile DH sires and dams, which can be used to produce viable offspring for the next generation. As shown by Bongers et al. (1999), the percentage of sterile fish in androgenetic progeny groups can be considerable (13 - 94%). Especially the low percentage of fertile females with a good egg quality can be regarded as a bottleneck. An example to illustrate this were the problems encountered during the production of the heterozygous isogenic strains in chapter 6. In total, 48 'presumed female' (= not male) DH fish were available. However, only ten of these showed female external phenotypic characteristics like a soft belly and a protruding uro-genital papilla. Together with seven fish selected purely for their estimated breeding value (EBV), these fish were injected with carp pituitary suspension (CPS). Of the ten injected individuals

with a feminine appearance, only two females produced viable larvae. The seven fish selected purely on EBV all proved to be sterile.

Although the fertility problems might be a reason to refrain from incorporation of a complete androgenetic homozygous generation in a selection programme, androgenesis can be a valuable reproduction tool within a large commercial selection programme. Besides the possible applications discussed earlier like estimation of breeding values, genetic load and G×E interactions, the most important application of androgenesis within a commercial selection programme is its use as a tool for faster dissemination of genetic progress in the breeding population to the commercial population and product protection (Van der Lende et al., 1998). In an ongoing breeding programme, sires with (a) desirable trait(s) present within the population can be selected for androgenetic reproduction. From the DH progenies of these sires, sires and dams can be crossed to produce all-male heterozygous isogenic strains, thus using both additive and non-additive genetic variation. Although the occurrence of heterosis depends on the trait and the species and lines used, significant heterosis has been found for an important trait like growth in a number of important aquacultural species like Atlantic salmon (Rye and Mao, 1998), rainbow trout (Wangila and Dick, 1996), Nile tilapia (*Oreochromis niloticus*; Bentsen et al., 1998; Marengoni et al., 1998) and common carp (Wohlfarth, 1993). The breeder can then market the strains with the best performance. The XY males within these strains are genetically uniform, but heterozygous, thereby limiting their value for further propagation. To enable the commercial breeder to preserve the genotypes of the founding parents for indefinite time, the selected sires and dams can be reproduced through andro- or gynogenesis, respectively, resulting in homozygous isogenic strains. However, given the fertility problems mentioned earlier, it is advisable to create both androgenetic and gynogenetic DH progenies. The latter can then be used to select the dams needed to produce the heterozygous isogenic strains.

4 Conclusions

Additive genetic factors have a strong influence on the stress-related cortisol response of common carp. A selection experiment resulted in isogenic strains of common carp with significant differences in the height of plasma cortisol concentrations at 20 min after onset of a cold shock after only one generation of subsequent family and individual selection. In a parallel experiment, aimed to study the stress response dynamics during a cold shock in different isogenic strains, a strain was discovered that consistently showed a more prolonged cortisol response during the cold shock compared to the other strains, indicating that the length of the response may have a

heritable character as well. The isogenic strains with the different cortisol response types will be valuable tools in future research into the stress response itself and its effects on other traits like growth, reproduction and health. This way, some of the problems related to the use of stress response as selection criterion in commercial breeding programmes in fish could be solved in the near future.

Residual heterozygosity was demonstrated to occur in androgenetic progenies, most likely due to maternal DNA fragments induced by the UV irradiation of the eggs. Improved control measures were implemented in the androgenesis procedure, but androgenetic progenies destined for further reproduction purposes should be screened for residual heterozygosity. Androgenetic reproduction proved to be a useful tool for dissection of phenotypic variance and heritability estimations for traits, especially in combination with selection experiments aimed at development of isogenic strains for this trait. Androgenesis might result in reduced fertility in female progeny, but the advantages are such that inclusion of androgenetic reproduction within larger commercial breeding programmes for faster dissemination of genetic progress and product protection should be considered as a promising option.

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Summary

The aim of the present thesis was to explore the genetic background of stress response in common carp (*Cyprinus carpio* L.) and produce homozygous and heterozygous isogenic strains with divergent stress responses. As stressor a rapid temperature decrease (= cold shock) was used. As a preparatory step, a number of experiments were carried out to: 1) investigate the validity of the cold shock as a stressor, 2) investigate the possible influences of environmental factors on the stress response, and 3) define a selection criterion for the selection experiment (**Chapter 2**). The stress response of common carp was studied by evaluating plasma cortisol, glucose and lactate after single or multiple rapid temperature drops (ΔT: 7, 9 or 11°C). All three amplitudes used induced a significant rise in plasma cortisol levels. Peaks occurred within 20 min after onset of the cold shock. No stress-related secondary metabolic changes were observed in any of the experiments described: plasma glucose levels remained unaffected and plasma lactate levels dropped. Carp of 60 days old showed a significant stress response, although plasma cortisol levels were lower than those observed in carp of 120 days. Furthermore, fish that had experienced multiple cold shocks showed an overall lower cortisol response than fish experiencing a single cold shock, indicating that habituation to this stressor occurred. Based on these results, the plasma cortisol concentration at 20 min after onset of the cold shock was set as selection criterion in our selection experiment and fish were tested at a minimal age of 100 days post hatching.

The first step in the actual selection experiment was the formation of the base population (**Chapter 3**). This base population was an F_1 cross between six sires from a wild strain originating from the Anna Paulowna (AP) polder and a highly domesticated homozygous E4 dam already present in our laboratory. The six sires, caught in the water system surrounding the Anna Paulowna (AP) Polder in The Netherlands, were characterised using allozyme and microsatellite markers. At the sMDH-A1,2* loci, an allele was found, which was previously only found in wild River Rhine and wild Vietnamese common carp. Microsatellite allele frequencies showed that these AP carp were significantly different from a group of carps originating from several different domesticated strains. Based on both allozyme and microsatellite data, the AP carp most likely originated from a wild or feral self-sustaining population.

Thirty-three randomly picked sires from these six E4×AP full-sib families (F_1) were androgenetically reproduced to create the F_2 generation, which thus consisted of 33 doubled haploids (DH) progeny groups (**Chapter 4**). These 33 DH progeny groups (566 individuals) were subjected to the 9°C cold shock, enabling us to estimate heritabilities for weight, length, condition factor (K), and plasma cortisol, glucose and lactate concentrations using Gibbs sampling and an animal model. Estimated

heritabilities for the morphological traits weight and length were 0.09 (90% Highest Posterior Density range: 0.03 – 0.17) and 0.11 (0.04 – 0.21), respectively. The condition factor (K), showed a medium heritability of 0.37 (0.20 – 0.62). Heritabilities for basal plasma glucose and lactate were 0.19 (0.10 – 0.33) and 0.56 (0.33 – 0.85), respectively. For stress-related cortisol increase, a high heritability estimate of 0.60 (0.37 – 0.90) was found. Although the height of this cortisol heritability has to be regarded with some reservation, due to confounding of some environmental effects with sire effects, the estimated heritability clearly shows that the stress response due to a cold shock is hereditary in the carp population used.

Because the model used to estimate the h^2 assumed a complete homozygous state of the DH individuals and to ensure that only homozygous individuals would be used for subsequent reproduction, all individuals within the 33 DH androgenetic progeny groups were analysed using 11 microsatellite markers to: 1) verify the homozygous status of the 566 androgenetic DH individuals, 2) analyse the microsatellite allele segregation, and 3) study possible association of microsatellite alleles with the phenotypic traits recorded (**Chapter 5**). In total, 92% of the androgenetic DH individuals proved to be homozygous at all 11 loci. Forty-three out of the 47 heterozygous individuals were heterozygous at a single locus only. This heterozygosity was probably due to DNA fragments caused by UV-irradiation of the eggs, although the maternal origin of the fragments could not be proved beyond doubt. Screening with 11 microsatellites also revealed two linkage groups, a segregation distortion at two microsatellite loci and possible association of some microsatellites with weight, length, stress-related plasma cortisol levels and basal plasma glucose levels. The success of the linkage and association study could be explained by a low recombination frequency due to high chiasma interference. This would imply a relatively short genetic map length for common carp.

Selection of individual fish from the 33 DH progeny groups based on the response at 4 months (chapter 4) was not possible. Therefore, three DH progeny groups with a high (H1-3) and three with a low (L1-3) mean plasma cortisol concentration were selected (**Chapter 6**). The 154 DH fish in these six groups were individually tagged, mixed and subjected to a second cold shock at an age of 15 months. For each individual fish, a breeding value was estimated (EBV_{15}) for stress-related cortisol using the animal model from Chapter 4 with a fixed h^2 of 0.60. Four homozygous sires (two high and two low) and two dams (high and low) were selected based on their EBV_{15} and used to produce four homozygous (HomIso) and eight heterozygous isogenic (HetIso) strains. These were used in two separate experiments to examine the genetic background of the

stress-related cortisol response. In both experiments, the strains were subjected to the 9°C cold shock at an age of 5 months. The ranking in plasma cortisol levels of the HomIso strains was identical to the ranking in EBV_{15} of the sires and the maximal difference of 350 nmol l^{-1} was similar to the expected difference based on these EBV_{15}'s. Differences between the HetIso strains were smaller than expected, and influence of non-additive genetic effects could not be detected ($P_{D\times S} = 0.14$). Estimated breeding values based on the performance of the androgenetic progeny (EBV_5) in experiment 1 and general combining abilities (GCA) of the sires and dams calculated in experiment 2 were positively correlated with the EBV_{15} (r not significantly different from 1), providing no evidence that the stress response at 5 and 15 months are different traits.

Apart from the isogenic strain used in Chapter 2, no complete profiles of the cortisol, glucose and lactate dynamics had been examined in other isogenic strains. Therefore, an additional experiment, parallel to the selection experiment, was carried out to investigate the 'complete' cortisol, glucose and lactate dynamics during the cold shock in four, readily available, isogenic strains at two different ages (**Chapter 7**). The experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. Significant dam and sire effects on the total amount of cortisol measured during the cold shock were found, but no significant dam × sire interaction effect. Although no significant difference was found between the cortisol response dynamics at 5 or 7 months, the results justify further research into that field. The observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related. Age did not have any apparent influence on either plasma glucose or lactate dynamics in both control and shocked fish.

Based on the results of the experiments described in this thesis, it can be argued that the best method to change the stress response of common carp would be through selective breeding (exploiting additive genetic effects) rather than through crossbreeding (exploiting non-additive genetic effects). The selection and the 'parallel' experiments resulted in several isogenic strains of common carp with at least two types of cortisol stress responses. Type I showed a relative short cortisol response with either a high or low peak at 20 min after onset of the shock. Type II showed a similar cortisol level at 20 min but no significant decrease in this level during the cold shock. These different isogenic strains will be valuable tools in future research into the stress response itself and its effects on other traits like growth, reproduction and health. This way, some of the problems related to the use of stress response as selection criterion in commercial breeding programmes in fish could be solved in the near future.

Residual heterozygosity was demonstrated to occur in androgenetic progenies, most likely due to maternal DNA fragments induced by the UV irradiation of the eggs. Improved control measures were implemented in the androgenesis procedure, but androgenetic progenies destined for further reproduction purposes should be screened for residual heterozygosity. Androgenetic reproduction proved to be a useful tool for dissection of phenotypic variance and heritability estimations for traits, especially in combination with selection experiments aimed at development of isogenic strains for this trait. Androgenesis might result in reduced fertility in female progeny, but the advantages are such that inclusion of androgenetic reproduction within larger commercial breeding programmes for faster dissemination of genetic progress and product protection should be considered as a promising option.

Samenvatting

Het in dit proefschrift beschreven onderzoek had tot doel om de genetische achtergrond van de stressrespons in de gewone karper (*Cyprinus carpio* L.) te bestuderen en isogene karperlijnen met verschillende stressresponstypen te produceren. Als stressor werd een snelle temperatuursdaling van het water (= koude schok) gebruikt. Ter voorbereiding van het selectie-experiment werden een aantal experimenten uitgevoerd om: 1) de geldigheid van een koude schok als stressor te onderzoeken, 2) de mogelijke invloeden van milieufactoren op de stressrespons te onderzoeken, en 3) een selectiecriterium te definiëren voor het selectie-experiment (**Hoofdstuk 2**). De stressrespons van de karper werd onderzocht door cortisol-, glucose- en lactaatconcentraties in het bloedplasma te meten na één of meerdere snelle temperatuursdalingen met verschillende amplitudes (ΔT: 7, 9 of 11°C). Alle drie de gebruikte amplitudes veroorzaakten een significante stijging van de cortisolconcentratie in het plasma. De hoogste waarden werden gemeten binnen 20 minuten na aanvang van de koude schok. In geen enkel experiment, dat in hoofstuk 2 beschreven is, werden secundaire (metabole) veranderingen waargenomen ten gevolge van stress: glucoseconcentraties in het plasma bleven gelijk en lactaatconcentraties daalden. Zestig dagen oude karpers lieten een duidelijke cortisol-stressrespons zien, maar de gemeten niveaus waren lager vergeleken met niveaus in 120 dagen oude karpers. Daarnaast lieten karpers, die meerdere koude schokken hadden ondergaan, een lagere cortisol-stressrespons zien dan karpers, die maar een enkele koude schok ondergingen, wat op gewenning duidt. Op basis van deze resultaten werd de cortisolconcentratie in het plasma op 20 minuten na aanvang van een 9°C koude schok gekozen als selectiecriterium binnen het selectie-experiment en werden de vissen in het vervolg getest op een minimale leeftijd van 100 dagen.

Als eerste stap in het selectie-experiment werd een basispopulatie geformeerd (**Hoofdstuk 3**). Deze basispopulatie (F_1) bestond uit een kruising tussen zes mannetjes van een wilde lijn uit de Anna Paulowna (AP) polder en een gedomesticeerd homozygoot vrouwtje van de E4 lijn, welke al in het broedhuis aanwezig was. Om de genetische achtergrond van de AP karpers te onderzoeken, werden de zes mannetjes gekarakteriseerd met behulp van allozym- en DNA-merkers (microsatellieten). Op het allozym locus sMDH-A1,2* werd een allel gevonden, dat voorheen alleen maar in wilde karpers uit Vietnam en uit de Rijn gevonden was. Daarnaast bleek uit de microsatellietanalyse, dat de zes AP karpers genetisch duidelijk verschillend waren van een groep karpers afkomstig uit verschillende gedomesticeerde lijnen. De zes AP mannetjes zijn daarom hoogst waarschijnlijk afkomstig uit een wilde of verwilderde populatie.

Uit deze zes E4×AP 'full-sib' families (F_1) werden willekeurig 33 mannetjes gekozen, die androgenetisch voortgeplant werden. Bij deze vorm van voortplanten wordt alleen het DNA van de vader doorgegeven aan de nakomelingen. De F_2 generatie bestond zodoende uit 33 families, elk met maximaal 40 dubbel haploid (DH) nakomelingen (**Hoofdstuk 4**). Al deze DH nakomelingen werden aan de 9°C koude schok onderworpen, wat ons in staat stelde erfelijkheidsgraden (h^2) voor gewicht, lengte, conditiefactor (K), en plasma cortisol, glucose en lactaat concentraties te schatten. De geschatte h^2 voor gewicht was 0.09 (90% betrouwbaarheidsinterval: 0.03 – 0.17) en voor lengte 0.11 (0.04 – 0.21). De conditiefactor (K) had een h^2 van 0.37 (0.20 – 0.62). De erfelijkheidsgraden voor basale plasma glucose- en lactaatniveaus waren respectievelijk 0.19 (0.10 – 0.33) en 0.56 (0.33 – 0.85). Voor stressgerelateerde cortisolconcentraties werd een hoge h^2 gevonden van 0.60 (0.37 – 0.90). Omdat de mogelijke invloed van enkele milieufactoren, zoals dag- en bakeffecten, niet volledig gescheiden kon worden van de genetische factoren, moet de hoogte van de cortisol h^2 met enige reserve bekeken worden. Deze h^2 toont echter wel duidelijk aan, dat de hoogte van de cortisol stressrespons in karper ten dele erfelijk bepaald wordt.

Omdat het model, dat gebruikt werd om de erfelijkheidsgraden te schatten, er van uitgaat, dat de gebruikte DH dieren volledig homozygoot zijn, werden alle 566 gemonsterde DH nakomelingen uit de 33 families gekarakteriseerd met 11 microsatellieten. Daarnaast werden deze gegevens gebruikt om de segregatie van de microsatelliet-allelen en mogelijke associatie (= koppeling) tussen deze microsatelliet-allelen en de gemeten fenotypische eigenschappen te testen (**Hoofdstuk 5**). In totaal bleek 92% van de geteste androgenetische DH dieren homozygoot te zijn op alle 11 loci. Drieënveertig van de 47 heterozygote dieren waren heterozygoot op een enkel locus. Alhoewel het niet volledig bewezen kon worden, werd deze heterozygotie waarschijnlijk veroorzaakt door de aanwezigheid van maternale DNA fragmenten, welke overgebleven zijn na UV-bestraling van de eieren. Verder werden twee koppelingsgroepen, afwijkende segregatiepatronen voor twee microsatellieten en mogelijke associatie van enkele microsatellieten met gewicht, lengte, stressgerelateerde plasma cortisolniveaus en basale glucoseniveaus gevonden. Het succes van de associatiestudie zou verklaard kunnen worden door een lage recombinatiefrequentie ten gevolge van (volledige) chiasma interferentie. Dit zou betekenen, dat de lengte (in centi-Morgan, cM) van de genetische kaart van de karper relatief klein is.

Omdat de androgenetische DH nakomelingen niet individueel gemerkt waren, was selectie op basis van de individuele stressrespons (Hoofdstuk 4) niet mogelijk. Daarom werden drie DH families met een hoge en drie families met een lage

gemiddelde cortisolconcentratie in het plasma geselecteerd (**Hoofdstuk 6**). De 154 vissen in deze zes families werden individueel gemerkt m.b.v. een transponder. Vervolgens werden ze gemixt en aan een tweede koude schok onderworpen op een leeftijd van vijftien maanden. Op basis van deze gegevens werd voor elke individuele vis een fokwaarde (FW_{15}) voor stressgerelateerde cortisol concentraties geschat op basis van de eigen prestatie (= stressrespons op vijftien maanden) en de prestatie van de androgenetische broers en zussen. Deze fokwaarden werden geschat met het model uit hoofdstuk 4 en een h^2 van 0.6. Op basis van deze FW_{15} werden vier homozygote mannetjes (twee hoge en twee lage) en twee vrouwtjes (hoog en laag) geselecteerd en gebruikt om vier homozygote (HomIso) en acht heterozygote isogene (HetIso) lijnen te maken. De lijnen zijn in twee verschillende experimenten gebruikt om de genetische achtergrond van de cortisol stressrespons verder te onderzoeken. In beide experimenten werden de lijnen onderworpen aan de 9°C koude schok op een leeftijd van vijf maanden. De rangorde in cortisolconcentraties van de HomIso lijnen was identiek aan de rangorde in FW_{15} van de verschillende vaders. Tevens was het maximale verschil in cortisol concentraties tussen de lijnen (350 nmol l^{-1}) gelijk aan het verwachte verschil op basis van de FW_{15}'s. De gevonden verschillen tussen de HetIso lijnen waren echter kleiner dan verwacht. Een significant effect van non-additieve genetische factoren (bijv. dominantie) kon niet vastgesteld worden. De geschatte fokwaarden (FW_5) voor de vaders op basis van de prestaties van de HomIso lijnen in experiment 1 en de individuele 'algemene combinatie geschiktheid' (= general combining ability) van de vaders en moeders, gebaseerd op de resultaten van de HetIso lijnen uit experiment 2, waren positief gecorreleerd met de FW_{15} van de verschillende ouders (Pearson's correlatie coëfficiënt (r) niet significant verschillend van 1). Deze bevindingen geven dan ook geen aanleiding om de stressresponsen op vijf en vijftien maanden als genetisch verschillende eigenschappen te beschouwen.

Afgezien van de isogene lijn, die in hoofdstuk 2 gebruikt werd, waren er geen volledige cortisol-, glucose- en lactaatprofielen beschikbaar van andere isogene lijnen. Daarom werden in een extra experiment, parallel aan het selectie-experiment, de 'complete' cortisol-, glucose- en lactaatprofielen in vier voorhanden zijnde isogene lijnen bestudeerd op twee verschillende leeftijden (**Hoofdstuk 7**). Uit dit experiment bleek, dat de profielen van de cortisol stressrespons (type I en II) stelselmatig kunnen verschillen tussen verschillende karpergenotypen. De totale hoeveelheid cortisol, die gemeten werd tijdens een koude schok, bleek sterk beïnvloed te worden door additief genetische factoren, maar non-additieve genetische factoren bleken geen rol van betekenis te spelen voor deze eigenschap. Hoewel er geen significant verschil werd

gevonden tussen de cortisolprofielen van vijf- en zeven maanden oude karpers, geven de resultaten voldoende aanleiding voor een vervolgonderzoek naar een mogelijk leeftijdseffect. De gevonden verschillen in glucose- en lactaatprofielen tussen de controle en geschokte vissen werden hoogst waarschijnlijk veroorzaakt door de dalende temperatuur zelf en niet door stress. Leeftijd had geen zichtbare invloed op de plasma glucose- of de lactaatprofielen, zowel in controle als geschokte vissen.

Op basis van de verkregen resultaten, lijkt selectie (exploitatie van de additief genetische effecten) een betere methode om de cortisol stressrespons van de karper te veranderen dan het kruisen van lijnen met een verschillende achtergrond (= 'crossbreeding'; exploitatie van de non-additieve effecten). De experimenten resulteerden in verscheidene isogene karperlijnen met tenminste twee cortisolresponsprofielen. Type I laat een relatief korte respons zien met een hoge dan wel lage piekwaarde op 20 minuten na aanvang van de koude schok, terwijl Type II een langdurige (hoge) cortisolrespons liet zien. Deze isogene lijnen, met de verschillende cortisolresponsprofielen, vormen een waardevol gereedschap voor toekomstig onderzoek naar de stressrespons zelf en zijn effecten op andere eigenschappen zoals groei, reproductie en gezondheid. Door dit vervolgonderzoek, zou het mogelijk moeten zijn om in de nabije toekomst enkele problemen, met betrekking tot het gebruik van de stressrespons als selectiecriterium in commerciële visfokprogramma's, op te lossen.

In de androgenetische DH nakomelingen bleek rest-heterozygotie voor te komen bij 8% van de geteste individuen. Deze rest-heterozygotie werd waarschijnlijk veroorzaakt door maternale DNA fragmenten, welke overgebleven waren na UV-bestraling van de eieren. Ondanks verbetering van de controle maatregelen tijdens het androgenetisch voortplanten van de mannetjes, moeten androgenetische nakomelingen, welke voorbestemd zijn als ouders te fungeren voor de productie van isogene lijnen, toch gecontroleerd worden op rest-heterozygotie. Androgenese bleek een goed gereedschap te zijn voor het ontleden van de fenotypische variatie en h^2-schattingen van verschillende eigenschappen, vooral wanneer dit gebeurd in combinatie met een selectie-experiment dat als doel heeft isogene lijnen te ontwikkelen. Androgenese kan resulteren in verminderde fertiliteit van voornamelijk vrouwelijke nakomelingen, maar de voordelen zijn dusdanig, dat het gebruik van androgenese binnen commerciële fokprogramma's, bijvoorbeeld voor een snellere verspreiding van de genetische vooruitgang en/of productbescherming, gezien moet worden als een veelbelovende optie.

NWO-ALW Programme:

Physiological strategies during acclimation to temperature-shock in fish

The role of the HPI-axis of the common carp during acclimation to rapid changes in temperature

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When fish face stressful conditions, the hypothalamus - pituitary - interrenal (HPI) axis is activated to enable the individual to cope with the stressor and to maintain homeostasis. A key function in the functioning of the HPI axis is attributed to the proopiomelanocortin (POMC)-derived hormones that are produced by the corticotrope cells in the pituitary pars distalis and the melanotrope cells in the pituitary pars intermedia. These hormones include adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and ß-endorphin. ACTH is a very potent stimulator of cortisol release by the interrenal cells in the head kidney, but in the Mozambique tilapia, also α-MSH has been reported to have corticotropic activity (Lamers et al., 1992), which can be potentiated by ß-endorphin (Balm et al., 1995). Cortisol is the end product of the HPI-axis and it reallocates energy away from investment activities to adaptation to stress; e.g. by restoring ionic balance. (reviewed by Wendelaar Bonga, 1997).

To further investigate the role of α-MSH and ß-endorphin in the stress response, we set up a series of experiments in which common carp (*Cyprinus carpio*) were subjected to a 9°C cold shock. As temperature influences virtually all physiological processes, it is expected that a sudden drop in ambient water temperature from 25°C to 16°C induces a stress response. At different time points after the onset of the temperature shock, blood and pituitary glands were taken and analysed for the amounts of cortisol, α-MSH and ß-endorphin.

We found that a 9°C cold shock induces a stress response indeed, as evidenced by rapid elevating plasma cortisol levels from 14 ± 13 to 247 ± 45 ng/ml (mean ± SD, n=10) after 20 minutes. Three hours after the start of the shock, the plasma cortisol concentration had declined to 63 ± 27 ng/ml. At this point, the shock was stopped and the water temperature was elevated to 25°C. Plasma cortisol levels subsequently

returned to basal levels (see also Chapter 2). There was no effect of the temperature shock on pituitary content and plasma concentrations of both α-MSH and ß-endorphin, indicating that there is no specific role for these peptides in the acute stress response in carp when a temperature shock is used as a stressor.

In additional experiments, we tried to determine the effects of the temperature shock on the brain. To this end, we applied functional Magnetic Resonance Imaging (fMRI) to study how a cold shock influences cerebral blood flow. Using this *in vivo* approach, we demonstrated that the blood flow decreased in the brain, but that the opposite was true immediately ventral to the pituitary gland. Whether this observation is a stress- or temperature-induced phenomenon is unclear at present. The rise in cortisol plasma levels and the changes in blood flow in the brain appear both to be very sudden effects rather then a gradual response in parallel to the decline in ambient water temperature. This may indicate that a temperature threshold exists in carp. Crossing this threshold would then trigger the stress response enabling the animal to adapt its physiological processes to the new ambient water temperature. For instance, we recently demonstrated that carp adapted to 15°C have double the amount of Na^+/K^+ -ATPase copies compared to 29°C-adapted fish (Metz et al., *in prep*).

In summary, a rapid drop in ambient water temperature induces a stress response in the common carp. The rise in plasma cortisol levels is likely an ACTH-mediated event, as both α-MSH and ß-endorphin are not involved in the stress response. The question as to whether there is a critical temperature below which carp have to completely change their physiological strategies to survive is currently under investigation.

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Temperature-induced stress and puberty in male common carp

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Stress is a ubiquitous feature of vertebrate life and has been shown to interfere with processes such as growth, immune response or reproduction. In fish, as in higher vertebrates, stress has been shown to cause an activation of the hypothalamic-pituitary-interrenal (HPI) axis, the equivalent of the mammalian hypothalamic-pituitary-adrenal (HPA) axis. In teleost fish, cortisol is the main glucocorticoid produced by the interrenals under influence of stress. Cortisol plays a key role in the restoration of homeostasis and is frequently indicated to be the major factor mediating the suppressive effect of stress on reproduction.

Puberty is the period covering the transition from an immature, juvenile to a mature, adult state of the reproductive system and may be more precisely defined as the developmental period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Stress has been shown to interfere at all levels of the BPG-axis and also effects of cortisol can be seen throughout the BPG-axis.

The aim of this study was (1) to confirm that the stress-induced activation of the HPI-axis is responsible for stress effects on reproduction and (2) at what levels the BPG-axis is affected.

Exposure of pubertal common carp to repeated cold shock caused an retardation of the testicular development, shown by a lower gonadosomatic index (GSI) of stressed fish, and histological analysis confirms that this is due to a retardation in spermatogenesis. This effect can be antagonised by co-treatment with RU486, a glucocorticoid receptor antagonist, indicating that cortisol mediates the deleterious effects of stress on testicular development.

Indeed, similar results can be obtained by prolonged cortisol treatment. Spermatogenesis was inhibited by the cortisol treatment and this was accompanied by lower plasma 11-ketotestosterone (11KT) levels and testosterone levels. *In vitro* studies demonstrate that cortisol has a direct inhibitory effect on testicular androgen production. 11KT has been shown to be an important hormone for spermatogenesis and currently we are investigating if the inhibitory effect of stress on testicular development is mediated by an inhibition of 11KT secretion.

On the level of the pituitary, prolonged cortisol treatment of pubertal carp resulted in a decrease in pituitary LH content, a decrease in sGnRHa-stimulated LH secretion *in vitro* and higher plasma LH levels at the end of puberty. Testosterone has been known to induce pubertal gonadotroph maturation, increasing the pituitary LH content and as a consequence the GnRH-stimulated release, but decreases plasma LH levels. Therefore we believe that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage but allows LH release.

In summary, stress inhibits pubertal development and this adverse effect is mediated by cortisol. Furthermore, cortisol acts directly at the level of the testis and thereby not only inhibits testicular development but indirectly also inhibits the gonadotroph maturation in the pituitary.

The influence of temperature-induced stress on the development and function of the immune system of the common carp *Cyprinus carpio* L.

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Stress induced immuno-suppression in fish is mostly attributed to actions of steroid hormones released upon activation of the hypothalamus-pituitary-interrenal (HPI)-axis (Weyts et al., 1999). As in mammals, the neuro-endocrine and immune system co-operate in a bi-directional way, sharing regulatory molecules and receptors. This project focuses on possible neuro-endocrine modulation of immune functioning through HPI-axis hormones during acute stress. Moreover, the interesting hypothesis is investigated if hormone secretion is regulated by interleukins from immune-cell origin.

Like mammals, fishes possess a complex and well developed immune system. Roughly the immune system can be divided in two types of responses: an innate or a-specific response and an acquired or specific response. In the innate immune response, phagocytic cells (macrophages and neutrophilic granulocytes) play a key role while in the specific response T- and B-lymphocytes are the important mediators.

So far, we studied the effects of acute temperature stress and the effects of cortisol, a major product of the HPI-axis, on the immune system. After repeated temperature shocks the relative number of B-lymphocytes in circulation, precursors of antibody producing cells, is significantly decreased. The decrease was even more pronounced after challenging the immune system with the T-cell independent antigen TNP-LPS (Engelsma et al., *in prep*). This drop in relative number can either be caused by the redistribution of cells to other body compartments or by programmed cell death, apoptosis. Previous work *in vitro* showed that especially activated B-lymphocytes are sensitive for cortisol leading to apoptosis (Weyts et al., 1998a). Immunisation of carp

with TNP-LPS induced lower antibody titers in the stressed group compared to the control. Together these results suggest impairment of the acquired immune system.

Cells of the innate immune system turned out to be less sensitive to cortisol. Neutrophilic granulocytes seem to be least affected by application of temperature stress. This is in agreement with previous *in vitro* experiments where neutrophilic granulocytes are even rescued from apoptosis by cortisol (Weyts et al., 1998b).

Cytokine molecules, like interleukin-1 beta (IL-1ß), play a pivotal role in the regulation of different processes within the immune system. Cells of the immune system release IL-1ß as a result of infection or tissue damage. Interestingly, as deduced from mammalian studies, they are important candidates able to affect the HPI-axis by altering the release of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH).

In fish, most interleukin molecules await identification but the IL-1ß sequences of several teleost fishes were recently elucidated. In the tetraploid carp we identified two IL-1ß genes (Engelsma et al., *in prep*). The two carp mRNA sequences share about 74% amino acid identity. Interestingly the IL-1ß2 sequence has an extensive polymorphism not found in the IL-1ß1 sequence. In contrast to some other fish species, in carp a constitutive expression of IL-1ß RNA is seen in predominantly the immune organs head kidney and spleen.

In vitro, in head kidney phagocytes, the IL-1ß RNA expression could be upregulated by stimuli such as for example lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria. In contrast, cortisol could inhibit the basal expression of IL-1ß RNA. However, when cells are pre-stimulated with cortisol or when cortisol is added simultaneously with LPS, cortisol could not inhibit LPS induced expression. Probably LPS can overrule the glucocorticoid receptor mediated inhibition via the nuclear factor-kB pathway (Engelsma et al., 2000). This would imply that cortisol cannot suppress the IL-1ß activation during infection.

Currently we are investigating the effect of recombinant IL-ß on immune functions, under stress and non-stress conditions. Together with our partners at the Department of Animal Physiology in Nijmegen we study the effects of IL-ß on release of pro-opiomelanocortin (POMC)-derived peptides and cortisol. To evaluate genetic differences in stress related immune modulation we will measure leucocyte activities and interleukin release in the two carp lines for high and low cortisol response (Tanck et al., this thesis).

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Curriculum vitae

Michael Willibrordus Theodorus Tanck werd op 24 september 1969 geboren te Lievelde, gemeente Lichtenvoorde. In juli 1987 behaalde hij zijn VWO diploma aan de r.k. scholengemeenschap "Marianum" te Groenlo. In datzelfde jaar begon hij met de studie Biologie aan de (toenmalige) Landbouwuniversiteit Wageningen. In augustus 1993 studeerde hij met lof af. Zijn afstudeervakken waren visvoeding (inclusief een stage bij het (toenmalige) BP Nutrition Aquaculture Research Centre, Stavanger, Noorwegen), visgezondheid en microbiologie. In de periode van oktober 1993 tot en met december 1995 was hij werkzaam als 'free-lance' onderzoeker, voornamelijk in opdracht van de vakgroep Visteelt en Visserij. In februari 1996 begon hij als Onderzoeker in Opleiding (OiO) bij dezelfde vakgroep binnen de onderzoeksschool Wageningen Institute of Animal Sciences (WIAS). Dat promotieonderzoek heeft geresulteerd in het thans voor u liggende proefschrift.