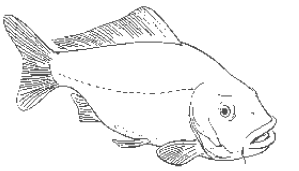
NWO-ALW Programme:

Physiological strategies during acclimation to temperature-shock in fish



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

M.W.T. Tanck and J. Komen

Fish Culture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

The aim of our research was to explore the genetic background of the stress response in common carp (*Cyprinus carpio* L.) and produce 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 investigate the validity of the cold shock as a stressor and define a selection criterion for the selection experiment. The stress response of common carp was studied by evaluating plasma cortisol, glucose and lactate after a rapid temperature drop (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. However, no stress-related related secondary metabolic changes were observed in any of the experiments described: plasma glucose levels remained unaffected and plasma lactate levels dropped. Based on these results, the plasma cortisol concentration at 20 min after onset of a 9°C cold shock was set as selection criterion in our selection experiment.

The first step in the actual selection experiment was the formation of the base population. 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. 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. These 33 DH progeny groups (566 individuals) were subjected to the 9°C cold shock, enabling us to estimate a heritability (h^2) for the height of the cortisol stress response. A high h^2 estimate of 0.60 was found, which 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. 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.

Selection of individual fish from the 33 DH progeny groups based on the response at 4 months 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. 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) for stress-related cortisol. Two homozygous sires (two high and two low) and dams (high and low) were selected based on their EBV 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 of the sires and the maximal difference of 350 nmol/l was similar to the expected difference based on these EBV's. Differences between the HetIso strains were smaller than expected, and influence of non-additive genetic effects could not be detected.

Apart from the isogenic strain used in the first experiments, 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. The experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. The observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related.

Based on the results of the experiments performed, 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 cross-breeding (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.

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

E.H. van den Burg, S.E. Wendelaar Bonga, and G. Flik
Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

When fish face stressful conditions, the hypothalamus-pituitary-interrenal (HPI) axis is activated to enable the individual to cope with the stressor and to realise homeostasis. A key function in the functioning of the HPI axis is attributed to 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 β-endorphin. ACTH is a potent stimulator of cortisol release by the interrenal cells in the head kidney, but in the Mozambique tilapia, also α-MSH has corticotropic activity (Lamers *et al.*, 1992), which can be potentiated by β-endorphin (Balm *et al.*, 1995). Cortisol is the end product of the HPI-axis and it reallocates energy away from investment activities, such as reproduction, growth and immune functioning, to adaptation to stress, e.g. by restoring ionic balance.

To 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 cortisol, α-MSH and β-endorphin contents.

Indeed, a 9°C cold shock induces a stress response, 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. There was no effect of the temperature shock on pituitary content and plasma concentrations of α-MSH and β-endorphin, indicating that there is no specific role for these peptides in the response of this fish to a temperature shock.

In subsequent experiments, we determined the effects of the temperature shocks on brain activity. 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 in the hypothalamic/pituitary region. Whether this observation is a stress- or temperature-induced phenomenon is unclear at present.

The rise in plasma cortisol 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 change itself is the stressor rather than the magnitude of the temperature drop. As ambient temperature influences virtually every process in poikilotherms, fish have to readjust their physiology. 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.*, 2001) to compensate for the lower activity of the enzyme at low temperatures.

In conclusion, a rapid drop in ambient water temperature induces a stress response in the common carp to counteract temperature-induced effects on its physiology. The rise in plasma cortisol levels is likely an ACTH-mediated event, as both α-MSH and β-endorphin are not clearly involved in this stress response.

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

M.Y. Engelsma, Van Muiswinkel, W.B. and Verburg-van Kemenade, B.M.L.
Cell Biology & Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, The Netherlands.

Stress induced immuno-suppression in fish is mostly attributed to actions of steriod hormones released upon activation of the hypothalamus-pituitary-interrenal (HPI)-axis. As in mammals the neuro-endocrine and immune system in fish 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 that 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. Previous work with cells cultured *in vitro* showed that especially activated B-lymphocytes were sensitive to cortisol, leading to programmed cell death, apoptosis. *In vivo*, after repeated temperature shocks the relative number of circulating B-lymphocytes (precursors of antibody producing cells) was significantly decreased. The decrease was even more pronounced after challenging the immune system (Engelsma *et al.*, in preparation). This drop in relative number can either be caused by the redistribution of cells to other body compartments or by apoptosis. In line with this, antibody titers of TNP-LPS immunized carp were lower in the stressed group compared to the control. Together these results suggest impairment of the acquired immune system after acute mild stress.

Cells of the innate immune system turned out to be less sensitive to cortisol. Of the leukocyte cell types neutrophilic granulocytes were least affected by application of temperature stress. This is in agreement with previous *in vitro* experiments where neutrophilic granulocytes were even rescued from apoptosis by cortisol.

Cytokine molecules, like interleukin-1 beta (IL-1β), play a pivotal role in

the regulation of different processes within the immune system. Cells of the immune system release IL-1β as a result of infection or tissue damage. Moreover, 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-1β sequences of several teleost fishes were recently elucidated. In the tetraploid carp we identified two IL-1β genes (Engelsma *et al*., in preparation). The two carp mRNA sequences share about 74% amino acid identity. Interestingly, the IL-1β2 sequence has an extensive polymorphism not found in the IL-1β1 sequence. In contrast to some other fish species, in carp a constitutive expression of IL-1β RNA was seen in predominantly the immune organs head kidney and spleen.

In vitro, in head kidney phagocytes, the IL-1β 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-1β RNA. However, when cells were pre-stimulated with cortisol or when cortisol was 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*., 2001). This might imply that cortisol cannot suppress IL-1β activation during infection.

Currently we are investigating the effect of recombinant IL-β 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-β on release of pro-opiomelanocortin (POMC)-derived peptides and cortisol. To evaluate genetic differences in stress-related immune modulation we will measure leukocyte activities and interleukin release in the two carp lines for high and low cortisol response.