INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Olfactory Imprinting in Sockeye Salmon (*Oncorhynchus nerka*)

by

Elmar Plate
Vordiplom, University of Bonn, 1992
Diplom, University of Hamburg, 1994

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

Dr. C.W. Hawryshyn, Supervisor (Department of Biology)

Dr. C.C. Wood, Departmental Member (Department of Fisheries and Oceans, Canada)

Dr. L.R. Page, Departmental Member (Department of Biology)

Dr. L.A. Hobson, Departmental Member (Department of Biology)

Dr. C. Barnes, Outside Member (Department of Earth and Ocean Sciences)

Dr. T.J. Hara, External Examiner (Department of Zoology, University of Manitoba)

Supervisor: Dr. C.W. Hawryshyn:

ABSTRACT

In the following study, I tried to link hormonal background conditions to successful olfactory imprinting in sockeye salmon by employing behavioural, endocrinological and electrophysiological experiments. In the initial experiments, sockeye salmon were exposed to potential imprinting odorants, with or without additional treatment with thyroid hormones, during several juvenile stages between fertilization and beyond the PST. After two years of rearing, these fish were tested for behavioural responses to test odorants in two behavioural arenas. Neither immature nor mature fish reacted behaviourally to the odorants that they had been exposed to previously. Therefore, exposure of juveniles to odorants did not lead to imprinting to those odorants under hatchery rearing conditions. In contrast, juvenile fish that were exposed to test odorants and treated with a combination of T_3 and T_4 (in all cases) or T_3 (in one case) the two most common forms of thyroid hormones, did exhibit an odorant recognition response two years later. However, the response differed between immature and mature fish. Mature fish were attracted to the imprinting odorant, whereas immature fish were repelled by the it. When immature fish were injected with GnRH before testing, their behavioural response was reversed. No behavioural response could be detected in fish that had been challenged with either T_3 or T_4 alone, in contrast to a combined treatment with both forms. Thus, I found evidence that a combination of T_3 and T_4 initiated imprinting and that GnRH motivated odorant recognition.

To examine the underlying hormonal processes, I first determined plasma thyroid hormone concentrations in sockeye salmon before and after hormonal challenges with

thyroid hormones or GnRH. In addition, the activity of the deiodinase enzyme that converts T_4 into the other possible forms of thyroid hormones was investigated in sensory and non-sensory tissues. The results suggested that only a combined T_3T_4 treatment increased the availability of both thyroid hormone forms in blood plasma, while a separate challenge with T_4 suppressed T_3 availability and vice versa. Moreover, the results provided evidence for deiodinase activity in the olfactory epithelium and the retina and demonstrated that GnRH can modulate the T_4 conversion process.

This information was helpful for planning and interpretation of the remaining experiments. Results obtained from a classical conditioning paradigm (heart-rate-conditioning), provided support for the hypothesis that GnRH lowers the threshold to an imprinting odorant and that the influence of GnRH was not restricted to an enhancement of motivation. To investigate whether hormonal action could also modulate the sensitivity of the peripheral olfactory system, electrophysiological responses from the olfactory epithelium (electro-olfacto-grams or EOGs) were recorded. The EOG results established that thyroid hormones and GnRH increased the EOG response of adult naïve (never imprinted to an odorant) fish, as did maturity. In the last experiments, I conducted EOG recordings on fish that had been imprinted at a juvenile stage. In summary, EOG recordings revealed that the imprinting process increased sensitivity to the imprinting odorant at maturity, while sensitivity in immature fish was decreased in comparison to non-imprinted fish. In combination with my behavioural results, this could explain why salmon do not enter their natal stream before they reach maturity. At maturity however, I also encountered desensitization to non-imprinting odorants, which might increase the ability to focus the olfactory system to the task of homing.

Examiners:

Dr. C.W. Hawryshyn, Supervisor (Department of Biology)

Dr. C.C. Wood, Departmental Member (Department of Fisheries and Oceans, Canada)

Dr. L.R. Page, Departmental Member (Department of Biology)

Dr. L.A. Hobson, Departmental Member (Department of Biology)

Dr. C. Barnes, Outside Member (Department of Earth and Ocean Sciences)

Dr. T.J. Hara, External Examiner (Department of Zoology, University of Manitoba)

Table of Contents

List of Tables

List of Figures

ACKNOWLEDGEMENTS

The completion of this dissertation was possible only as a combined effort of many people. I would first like to thank my supervisor Dr.Craig Hawryshyn and the committee member Dr. Chris Wood. They took me on as a scholar and had faith in my enthusiasm regardless of a lack of detailed understanding of the subject, when I started out. They gave me the freedom to follow my scientific ideas and provided firm guidance when necessary. Without them, I would not have finished this dissertation.

Dr. Louise Page, Dr. Lou Hobson and Dr. Chris Barnes guided my scientific efforts as committee members and I am very thankful for the critical and positive input that I needed and received.

Jim McGinnis went far beyond the call of duty when he assisted me in the maintenance of my 17000 sockeye children throughout my experimental work. He became a good friend and I was adopted as a grandchild. The warm welcome that he and his wife Betty extended to me made me feel at home in Canada.

Ron Traber assisted in the construction of all set-ups, tanks, roofs, wiring and plumbing in a positively mentoring way. My colleague Craig McDonald made the life on the dark side much easier and regardless of the often long hours he kept his sense of humour and his amazing ability to be a creative thinker, never shy of ideas for a new experiment. I thank my other UVic colleagues Shelee Hamilton, Ted Allison and Garnet Martens for their permanent positive feedback on my work and their friendship. Pat Kerfoot, the most friendly and helpful technician I have ever encountered, provided crucial help in the construction of the electrophysiological rig, so did Gordon Davies. Eleanore Floyd, in her knowledgeable, positive and encouraging way, never became impatient and therefore allowed me to conduct the first three years of my research away from the university.

For financial support, I have to thank the Department of Fisheries and Oceans, Canada the Git'Ksan Wet'Suet'En Watershed Authorities for a four year scholarship and Chris Barnes and Charly Muldoe for the successful co-operation. Moreover, the Science Council of British Columbia provided me with a one year GREAT scholarship.

Finally I have to thank my mother Rosemarie Plate and my partner Helen Truran to encourage me when I was in a desperate mood, not easy to deal with and needy of love and a kick in the ....

DEDICATION

To all the people who made my life such a happy one

Chapter I: General Introduction

1. Background

a. Life cycle of sockeye salmon:

A life history (Figure 1.1.) (for reviews, see: Groot & Margolis, 1991) that utilizes two environments, ocean and freshwater streams, allows salmon to take advantage of beneficial attributes of both environments. Without human influence this life cycle seems well adapted to the environments occupied. Highly oxygenated gravel beds of the spawning streams provide protection from predators and facilitate survival during the vulnerable stage of egg incubation (Foerster, 1968; Burgner, 1991). Once hatched, the juveniles enter the alevin stage until the yolksac is absorbed and the fish swim-up out of the gravel and start to feed. Juveniles, now free-swimming, leave the natal stream and venture into a lake to establish a planktivorous lifestyle in schools. At this stage, sockeye salmon must internalize information about the chemical composition of their environment (imprint) for the first time, since they will not enter their natal stream again before they migrate towards the ocean. After one or more years in the lacustrine environment, depending on their overall condition and their genetic predisposition, juvenile sockeye undergo the parr-smolt-transformation (PST) and begin their seaward migration (Hoar, 1976, 1988). During this downstream migration, sockeye salmon constantly experience new environments and imprint for the second time to recall the olfactory characteristics of the changing environments. This olfactory information will be used for homing orientation, once maturity approaches.

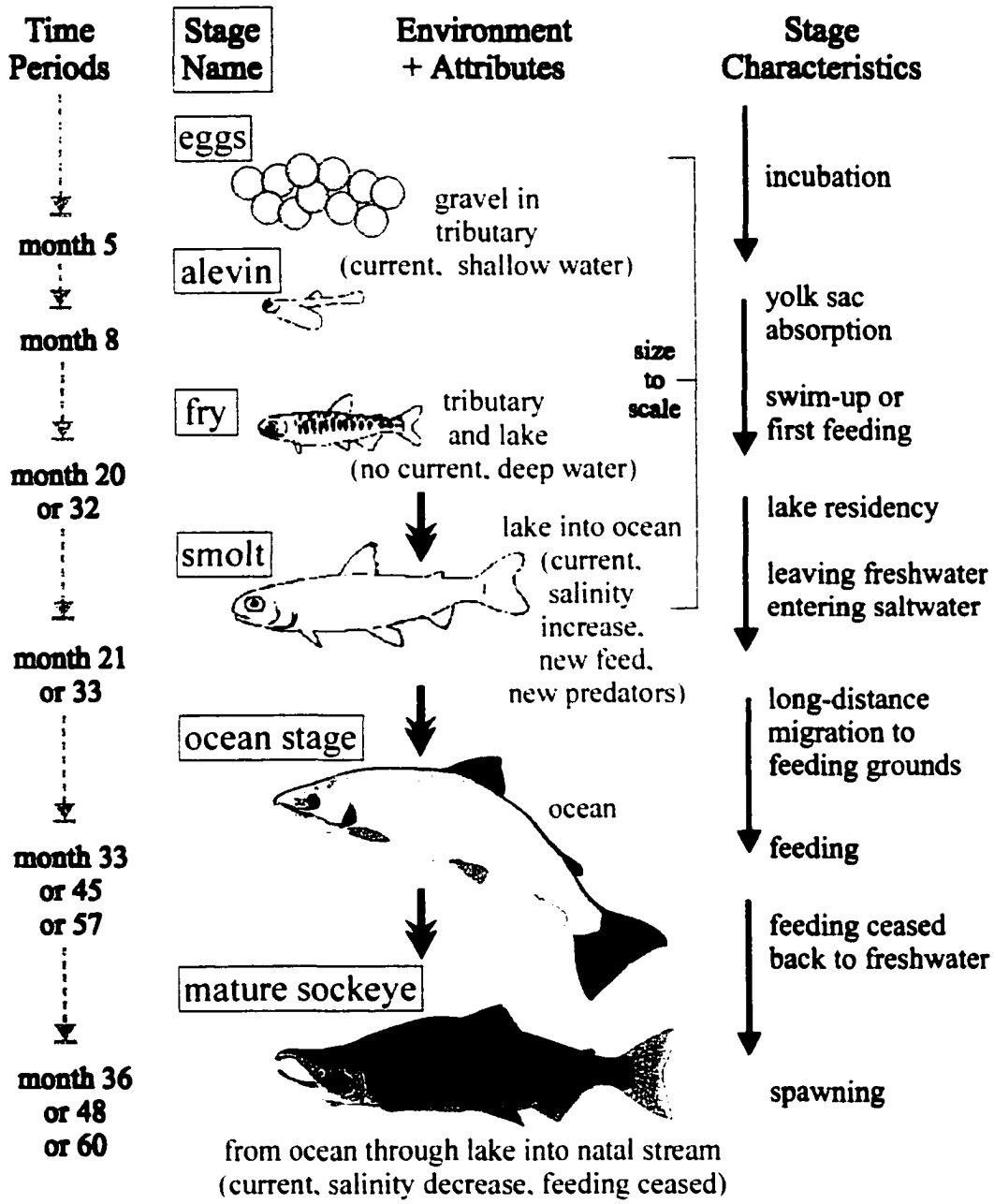


Figure 1.1.: Time course, stage names, environments experienced and their attributes and descriptive characteristics of each stage in the life cycle of sockeye salmon. Drawings of the first four stages (egg, alevin, fry and smolt) are to scale. Double arrows show times of migration.

The rich feeding grounds of the ocean allow sockeye salmon to grow to larger size than they could by remaining in fresh water. Typically after one to four years a cascade of hormonal processes initiates maturation and the fish migrate back to their natal stream. The physical-chemical signature of the natural environments experienced at juvenile freshwater stages is now used to locate first the rearing lake and then the natal stream (Hasler & Wisby, 1951). Upon reaching the natal gravel beds, spawning occurs and subsequent death completes the life cycle.

b. Sensory systems used in migratory behaviour

Limited by the capabilities of our own senses, humans often fail to recognize the sensory systems that animals use to navigate through environments that appear bare of obvious orientational cues. Prominent examples of migratory behaviour guided by sensory capabilities that are currently not well understood include: migratory birds orientating in overcast conditions at night; whales migrating through oceans distant from any landmarks (Dingle, 1996); and the long-range ocean migration of Pacific salmon. The economic importance of salmon led to an early interest in details of their migratory routes and the sensory mechanisms used to guide the migratory behaviour.

In the ocean, the following sensory systems have been suggested to guide migratory behaviour in salmon: Orientation towards currents was proposed by Westerberg (1984), Doving et al. (1985) and Quinn & TerHart (1987). Within this hypothesis currents not only serve as constant orientational cues, but also carry fish towards their home stream and thus decrease the energetic investment necessary to

accomplish the long distance oceanic migration. The potential use of magnetic compass orientation was implied by Quinn (1982) based upon evidence for compass orientation in freshwater experiments. Blackbourn (1987) suggested orientation towards temperature gradients and Quinn (1980) suggested the possibility of orientation to local magnetic fields. Local magnetic field orientation, in contrast to magnetic compass orientation, is based upon the detection of naturally occurring irregularities in the earth's magnetic fields around mountain chains on the ocean floor. The nature of the sensory system used to perceive compass direction or irregularities of magnetic fields is still unknown and therefore a matter of intensive research (Wiltschko et al., 2000). Another mechanism that has been suggested to guide long-distance migrations in the ocean, is the ability of salmon to perceive the orientation of polarized light (Groot, 1972; Hawryshyn, 1992).

When salmon re-enter fresh water, the recognition of olfactory stimuli seems to be the dominant sensory modality that guides the final part of homing migration (Hasler & Wisby, 1951; Hasler & Scholz, 1983).

c. The role of olfaction: Summary of behavioural experiments

The life history of salmon allows for easy access to mature or maturing fish in fresh water. This accessibility provides opportunities for behavioural experiments and thus research on the role of olfactory perception for homing has a long history (Scheer, 1939; Hara, 1970; Hasler, Scholz & Horall, 1978; Cooper & Hirsch, 1982; Stabell, 1984; Quinn, 1993; Nevitt & Dittman, 1998; Barinaga, 1999). Sensory systems can be experimentally altered and effects can be observed directly or with the help of radio-

telemetry. Initial experiments on the effect of olfactory impairment on correct homing in freshwater were conducted by Hasler & Wisby (1951, Wisby & Hasler.1954) who established a link between an intact olfactory system and the precision of homing in coho salmon. Hasler, Scholz, and associates Horall, Cooper and Madison focused on the basic mechanisms of imprinting and developed a theory of olfactory imprinting in salmon (Hasler, 1966; Scholz et al., 1976; Hasler et al., 1978; Hasler & Scholz, 1980). Their seminal studies relied on the use of morpholine and phenyl-ethyl-alcohol as artificial imprinting substances and the subsequent release of marked coho salmon smolts into tributaries of Lake Michigan. Returning mature fish, in this study, were shown to ascend into streams treated with the imprinting substance and the imprinting odorant proved to be more important than the release site of the fish (Madison et al., 1973). Thus, Madison et al.(1973) demonstrated that salmon explicitly use olfactory stimuli to locate their natal stream and the olfactory imprinting theory was born. Hasler and Scholz (1980) recommended the use of the artificial imprinting procedure for the conservation of endangered salmon stocks. They proposed to lead salmon that had been exposed to morpholine at the smolting stage into adjacent non-natal streams by scenting the non-natal streams with morpholine. It was hoped that this approach could be used to colonize additional river systems without the large expense of conventional enhancement programs. In the following years, the simplicity of the olfactory imprinting theory was questioned. Hasler and colleagues (1983) had assumed that naïve salmon were neither attracted to nor repelled by morpholine since natural attraction or repulsion to an odorant can mask behavioural reactions based on imprinting. This suggestion was questioned by Mazeaud (1981,1982), who showed that salmon were attracted to morpholine without

previous exposure. Another morpholine imprinting experiment in a hatchery on the Oregon coast failed to corroborate the original results (Rehnberg et al., 1985). Since then, morpholine has not been used in imprinting experiments. Dittman and Quinn (1996) used phenyl-ethyl-alcohol as an imprinting odorant to show that successful olfactory imprinting was restricted to the PST stage, which confirmed earlier findings by Hasler and Scholz (1983). Physiological conditions that allow for olfactory imprinting were further clarified by electrophysiological and endocrinological research, as described below.

d. The role of olfaction: Summary of electrophysiological experiments

The olfactory imprinting theory for salmon as proposed by Hasler and colleagues (Cooper & Hasler, 1974; Hasler & Scholz, 1983) was questioned when electrophysiologist Hara (1974) examined the effectiveness of morpholine as an olfactory stimulant. Hara found morpholine to be non-stimulatory for rainbow trout at the concentrations used in imprinting experiments conducted by Cooper & Hasler (1974,1976), Cooper & Scholz (1976) and Scholz et al. (1978a). A high morpholine concentration of 0.1 M was also found to inhibit the EEG (electroencephalogram) response to an odorant (L-Serine) that was normally highly stimulatory at the level of the olfactory bulb (Hara, 1974). In addition, Hara (1976) revealed that high concentrations of morpholine increased the pH of the surrounding medium, which affects the olfactory transduction processes, and can irreversibly inhibit the olfactory responsiveness to other odorants (Hara & Brown, 1979). Within the same set of experiments, Hara and Brown could not find a manifestation of the imprinting process in the neuronal transduction

process when recording EEG responses to morpholine. Rainbow trout that had been exposed to morpholine 12 months prior to testing did not display an alteration in their EEG responses to morpholine when compared to non-exposed control fish. However, it remained unclear whether their fish had failed to imprint, whether their electrophysiological recording technique was unsuitable to detect imprinting based sensitivity changes, or whether sensitivity to the imprinting odorant would increase only under particular physiological conditions. In response to Hara's and Brown's findings, Cooper (comment and response published in Cooper, Hara & Brown, 1982) suggested that reproducibility of imprinting responses was highly dependent on using the proper developmental stage. This way, Cooper indirectly pointed to the possible regulatory role of hormones for successful imprinting of juvenile fish and odorant recognition in homing, mature salmon.

e. Do hormones control imprinting and odorant recognition?

In a series of experiments Hasler and Scholz (1983) gathered evidence that suggested hormonal control of the imprinting process. First, blood plasma thyroid hormone concentrations were found to be at their highest level in coho salmon smolts during their migration from fresh water into saltwater. Subsequently, Hasler and Scholz (1983) compared odorant recognition performance of coho salmon that had been simultaneously exposed to phenyl ethyl alcohol and thyroid stimulating hormone (TSH) ten months earlier with groups that had been exposed to the olfactory stimulus without the hormone. Neither of the two treatment groups reacted behaviourally to the imprinting odorant before maturity. At maturity, the group that was exposed previously to both,

odorant and TSH, showed a behavioural response to the imprinting odorant. This suggested that thyroid hormones facilitated juvenile imprinting and that the stage of maturity allowed the fish to recognize the imprinting odorant. Within the array of hormones that initiate maturation, GnRH (gonadotropin-releasing hormone) was investigated for its possible role during migratory behaviour by Sato et al. (1997). They demonstrated that homing behaviour was intensified in lacustrine sockeye salmon following treatment with GnRH. GnRH was further implicated in the homing process when Okubo et al.(2000) showed the expression of GnRH receptors in the olfactory epithelium of the migratory Japanese eel (*Anguilla japonicus*) and Eisthen et al. (2000) demonstrated neuromodulatory capabilities of GnRH on olfactory receptor neurons (ORNs). Therefore, GnRH may act to enhance swimming motivation or as a neuromodulator at the level of the ORN.

f. Importance of precision in homing

We now know that the large majority of sockeye, coho and chinook salmon home to their natal stream and only a small number of fish stray into nearby streams (Hasler 1966, Harden-Jones 1968;Quinn et al., 1987; Quinn 1993). Why is precise homing of such importance? Failure to spawn in the natal stream can reduce fitness because salmon typically develop adaptations that enhance reproductive success in their natal stream (Ricker, 1972; Taylor, 1991). Considerable evidence indicates that indigenous fish populations are more successful in reproduction than transplanted fish (Ricker, 1972; Reisenbichler, 1988). From this perspective, the ability to locate the home stream precisely is itself a behavioural adaptation of great importance. Therefore it is not

surprising that the system of olfactory imprinting leading to precise homing is a highly developed, rather complex phenomenon that is intriguing to study.

2.Questions of seminal interest to my thesis

Hasler and Scholz (1983) found that coho salmon failed to imprint when they were exposed to morpholine subsequent to smoltification. This observation implies a temporal window for olfactory imprinting that is restricted to the period prior to or during parr-smolt-transformation (PST). If it is possible to artificially induce imprinting of salmon after PST, facilitated by exposure to thyroid hormones, one could exclude the possible influences of the other physiological PST changes on the imprinting process and clarify the role of thyroid hormones. Therefore, salmon within my study were exposed to imprinting odorants and thyroid hormones prior to and after the PST to investigate whether imprinting could occur independently from this developmental stage. My rationale was to clarify whether the developmental stage or the hormonal status experienced during exposure to an odorant would be more important for successful imprinting. Two behavioural arenas were employed to test for behavioural responses of three-year-old immature and mature sockeye salmon to odorants that the fish had been exposed to at juvenile stages.

The two main forms of thyroid hormone are T_3 and T_4. The physiologically inactive T_4 has been suggested as the agent that initiates olfactory imprinting (Scholz,1980; Dittman et al., 1996). In contrast, T_4 exposure has been shown to decrease the amplitude of neuronal olfactory responses in late parr of Atlantic salmon (*Salmo salar*) (Morin et al., 1997). To explain this contradiction, it has to be emphasized that T_3

equals T_4 minus one iodine group and that T_3, due to greater receptor affinity for nuclear binding sites of sensory cells, is assumed to be the physiologically active form of thyroid hormone (Eales, 1995). Conceivably, desensitization of the olfactory system following T_4 treatment maybe the consequence of a T_3 decrease that occurs in response to T_4 challenges (Morin et al., 1995, 1997). The possible interaction between the two forms of thyroid hormone and their physiological role in the juvenile imprinting process is therefore unresolved and clarification requires more research. In Chapter II, I address the role of the two forms of thyroid hormone in behavioural odorant recognition tests and in chapter IV and V a classical conditioning paradigm and electrophysiological experiments are utilized to elucidate possible effects of the two forms of thyroid hormone.

The status and interaction of the two forms of thyroid hormone and gonadotropin-releasing hormone (GnRH) in blood serum and at the level of peripheral sensory tissues like the olfactory epithelium and the retina, is not well understood. Therefore I monitored the thyroid hormone status in different sensory and non-sensory tissues and recorded possible changes in response to GnRH challenges in Chapter III of this study. In this manner, I tried to investigate the possible direct or indirect role of GnRH in peripheral control of thyroid hormone responsive tissues, which might influence the processes of olfactory imprinting and homing-odorant recognition.

The basis for odorant recognition during homing by mature fish is successful imprinting early in development and the storage of the imprinted information over several years. Nevitt et al. (1994) provided first evidence for imprinting related peripheral sensitization using single-cell patch clamp preparations of ORNs. In continuation of Nevitt et al.'s work, Dittman et al (1997) monitored increased activity of

guanylyl cyclase in ORNs in response to an imprinted odorant and thus provided a possible explanation for imprinting based peripheral sensitization. Nevertheless, these results are difficult to explain since ORNs continue to turn over during adult life (Farbman, 1994) and their sensitized state has to be manifested in a higher center of the olfactory system. In Chapter V of this study, I therefore investigated peripheral retention of olfactory information during imprinting in electro-olfactograms (EOGs). EOGs are typical examples of compound action potentials (CAP). They represent the combined receptor potentials of many olfactory receptor neurons recorded at the surface of the olfactory epithelium (Ottoson, 1971). Peripheral sensitization initiated by the imprinting process should therefore become visible when the EOG responses of imprinted and non-imprinted fish are compared. It should further be possible to detect general sensitization in non-imprinted fish based on the onset of maturity or hormonal action in EOGs.

The overall strategy of my thesis research was to take an integrative approach to investigate mechanisms of olfactory imprinting in juvenile fish. The success of the juvenile imprinting strategy was measured in the behavioural response of adult fish to the imprinting odorant. To facilitate my research, three generations of test fish were reared from fertilization until death in a controlled hatchery environment, making it the only study of its type. Although this approach was time and labour intensive, environmental conditions of the fish could be controlled at all times and clear causal links between treatments and behavioural, endocrinological and electrophysiological responses could be made.

Chapter II: Odorant recognition based on olfactory imprinting in sockeye salmon (*Oncorhynchus nerka*)

II.1. Introduction:

In this study, two behavioural tests were employed to establish the hormonal background conditions that facilitate the internalization of odorant stimuli (imprinting) in juvenile sockeye salmon. Sockeye salmon were chosen as the experimental species because of their unique life history, which typically differs from the life cycle of the other four North American Pacific salmon species by the inclusion of a one to two year period of residence in a lake (see also Figure 1.1.) (Foerster, 1968; Burgner, 1991). Sockeye salmon migrate twice in freshwater before they enter the ocean. First, when they leave their natal stream for the rearing lake and second, when they proceed to the ocean by migrating from the rearing lake to the estuary of a river system during the parr-smolt transformation (PST). Since it is commonly assumed that salmon imprint during times of juvenile migration (Hasler & Scholz, 1983, Dittman et al., 1994), sockeye salmon should imprint during at least two periods in nature. The natural necessity of multiple imprinting should make sockeye salmon a well-suited organism for experimental imprinting studies at different stages in captivity.

Within the anadromous life history of sockeye salmon, many adaptive processes are necessary to endure the variety of environments encountered. For the most part, these processes are under hormonal control (Hoar, 1976, 1988; Dingle, 1996). Thyroid hormones for example initiate physiological adaptations including silvering of the body, an increase in salinity tolerance, a heightened hemoglobin complexity to compensate for

lower oxygen saturation, stimulation of the overall metabolism (Hoar, 1988) and adaptation of the visual system to differences in changing light environments (Browman & Hawryshyn, 1992). In nature, thyroid hormones typically peak during PST but they have also been shown to increase at first feeding in sockeye salmon (Tilson et al., 1994, 1995). It has been suggested that environmental cues act via the hypothalamus pituitary axis to initiate the increased production and circulation of thyroid hormones. The hypothalamus is neuronally connected with the mesencephalon and the optic tecta, where multimodal sensory input is processed (Hazon & Balment, 1998; Wulliman, 1998). Environmental cues include: changes in photoperiod (Morin et al., 1997), lunar phase (Grau et al., 1981), temperature (Hoar, 1976) and current (Youngson & Simpson, 1984). In the monotonous hatchery environment, the aforementioned physical parameters do not change and the less precise homing observed in hatchery-reared salmon has been attributed to this lack of sensory stimulation (Dittman & Quinn,1996). In conclusion, migratory restlessness, sensory stimulation and increasing thyroid hormone plasma levels seem to co-occur at stages of olfactory imprinting in nature but not in hatchery rearing conditions. While this temporal correlation is suggestive of a causal relationship between the three factors and imprinting, only indirect experimental evidence for the possible causal relationship exists. When Scholz (1980) and Hasler and Scholz (1983) stimulated the thyroid gland of hatchery-reared juvenile coho salmon by thyroid-stimulating hormone (TSH) instead of environmental cues, they improved the homing precision of these fish at maturity. TSH stimulates the thyroid gland to produce T_4, the physiologically inactive form of thyroid hormone, which has to be converted to the physiologically active T_3. Moreover, artificially increased T_4 plasma concentrations can lead to a decrease of T_3

plasma levels in Atlantic salmon (Morin et al.,1995, 1997). Thus it is unknown whether T_3, T_4, a combination of both forms of thyroid hormones or other processes related to the chosen developmental stage were responsible for the facilitation of the imprinting process (Scholz, 1980; Hasler and Scholz, 1983).

Therefore, my study was designed to specify whether odorant exposure at different developmental stages or the hormonal status of the fish trigger olfactory imprinting.

The effectiveness of my juvenile olfactory imprinting protocol was evaluated two years after imprinting in two behavioural arenas. To monitor subtle behavioural changes to odorants, I used the behavioural trough (Figure 2.2.) (Jones & Hara, 1985). To determine whether odorant recognition would motivate fish to overcome a low-profile waterfall, behaviour was monitored in an experimental set-up that combined natural aspects of a spawning stream, the Y-maze waterfall (Figure 2.3.). In combination, the two set-ups were used to correlate the conditions at juvenile odorant exposure with behavioural responses of immature and mature adult fish to the possible imprinting odorants.

The choice of odorant used to imprinting fish experimentally is important. Hasler and Scholz (1983) were criticized because it was never clarified whether the imprinting odorant that they used (morpholine), was a non-specific irritant or a potent stimulant for the olfactory system (Hara, 1974, Cooper et al, 1982). Therefore, I defined suitability of an odorant for imprinting purposes in my experiments in the following terms: Sockeye salmon had to be able to either perceive the odorant, as proven in electrophysiological tests, or the odorant had to occur naturally at the used concentration. Moreover, fish were

not supposed to be naturally attracted to or repelled by the chosen odorants. The following representatives from three different groups of odorants were chosen:

1. L-alanine and L-glutamine represented amino acids, which are naturally occurring in a large variety of forms and are emitted directly from fish and decay of organic matter. Amino acids are also widely used as standard odorants in electrophysiological recordings from the peripheral olfactory system in fishes (Hara et al., 1984).
2. Cholic acid was the representative of the bile acid group, which is naturally emitted from fish and can be detected at very low concentrations (Hara et al., 1984).
3. $MnCl_2$, $BaCl_2$ and $CuCl_2$ were chosen to represent metal ions or bivalent cations that are ubiquitously occurring in nature. $MnCl_2$, for example, was found at concentrations of 10^{-6} M -10^{-7} M in Fulton River, a major spawning tributary of sockeye salmon running into Babine Lake and one of the streams that eggs for this study were collected from. Moreover bivalent cations can substitute calcium ions in the bony tissue of salmon species and their composition can be matched to the composition of natal streams to reveal the freshwater origin of fish that are caught in the ocean (Mulligan et al., 1983). Thus, the compositions of molecules, which are substituting calcium ions, are a representation of the unique bivalent cation composition of a stock's natal stream. Electropysiological recordings, testing for responses to bivalent cations had not been undertaken before this study.

Successful imprinting to these odorants was defined as a behavioural response of sockeye salmon in two different behavioural arenas two years after last exposure to the imprinting odorants. This experimental design also allowed for an assessment of the influence of maturity and the maturity-related hormone gonadotropin-releasing hormone (GnRH) for the initiation of behavioural responses to potential imprinting odorants. The fact that three generations of fish were reared from fertilization until maturation allowed me to standardize background conditions throughout ontogeny, making this study the first one of its kind.

II. 2. Materials and methods

1. General materials and methods for all chapters:

Egg sampling, incubation and rearing

Thirty spawning sockeye and kokanee males and females were sampled from Pierre Creek, a tributary of Babine Lake located in northern British Columbia, Canada, in August of 1995. Kokanee salmon were paired with kokanee and sockeye salmon with sockeye and eggs from one female were fertilized with the sperm from a single male in water.

For incubation, eggs were transported (20 hours at a temperature of 3-5°C) to Rosewall Creek Experimental Hatchery (Fanny Bay, Vancouver Island, British Columbia, Canada). During incubation, in standard under-current incubation trays (Heath trays), the temperature was held at 8.5 ± 1°C. Eggs of individual pairs were kept separate until the eyed stage, to trace mortalities to genetic crosses. Subsequently, eggs of individual pairs within the sockeye or kokanee subspecies were combined for easier maintenance. Once the larvae had reached the late alevine stage (yolksac larvae) they were moved into buckets with a screened bottom floating in holding tanks (30 cm diameter, 50 cm deep) in a building with natural photoperiod. The floating buckets served to keep the fish away from feces that constantly accumulated at the bottom of the tank and simplified tank maintenance. When the yolksac was visibly absorbed, food (Ewos, Vextra, Starter Food) was offered every hour until swim up, at which point juveniles feed actively.

Rationale for using sockeye and kokanee salmon

With warmer water temperatures and higher metabolism and feed intake in a hatchery environment during the winter months, sockeye salmon mature at the end of year three, which is one year earlier than in the wild. A small percentage of precocious sockeye males or "jacks", mature at the end of year two. Under the same conditions kokanee salmon, the resident or non-ocean going form of *Oncorhynchus nerka*, reach maturity at the end of their second year (Wood & Foote, 1996). Combined, the two forms of sockeye salmon allowed me to test mature fish over a period of two to four years. Thus, temporal flexibility was gained and the project was not based on only one testing season when mature fish were available.

Thyroid hormone feed treatment

Feed was treated with either T_3 (12 ppm) or T_4 (120 ppm) independently or T_3 (12ppm) and T_4 (120 ppm) combined. Thyroid hormones were dissolved in ethanol and sprayed onto pellets whereas feed for the control groups was sprayed with ethanol only. Pellets were frozen for storage once the ethanol was absorbed or evaporated. The fish were fed 1-2% of their average body weight on a daily basis by hand feeding or through automatic feeders.

Fish rearing and GnRH treatment

During odorant and hormone treatment sockeye were reared in three-foot tanks (10 L • min^{-1} flow, well water 8.5 ± 0.5°C) until September of 1998 (age, three years) when approximately 50% matured. Only completely silver fish that did not show any

morphological signs of maturity (i.e. darker body colouration, humpback) were chosen for the GnRH injection. GnRH analogue (20 µg • kg $^{-1}$ body weight of D-Ala-6, des Gly^{10} ethyl amide) dissolved in a 9‰ saline was injected into epaxial musculature twice, 36 h and 12h before blood samples were taken or before the fish were tested behaviourally. J.E. Rivier (Salk Institute, La Jolla, California) generously donated the GnRH analogue. Injection number one and two were assumed to stimulate pituitary GTH synthesis and secretion respectively (N. Sherwood personal communication).

Table 2.1.: Concentrations used for odorant exposure at juvenile stages to achieve imprinting and in three-year-old fish to test for behavioural responses.

Odorant	Molecular weight in [g • M^{-1}]	Concentration
$MnCl_2$	125.8	10^{-6} M
$BaCl_2$	208.3	10^{-6} M
$CuCl_2$	134.5	10^{-6} M
L-glutamine	146.1	10^{-5} M
L-alanine	89.01	10^{-5} M
cholic acid	408.6	10^{-8} M

Odorant exposure of juvenile sockeye salmon without hormonal treatment

Juvenile sockeye were exposed to odorants during the following stages:

1. First feeding until three months after PST: This period of odorant exposure began at the time when the yolk sac was visibly absorbed and fish were starting to swim-up and feed. It entailed the fry-parr and the PST stage and ended three months after first morphological signs of PST. Overall, fish were exposed to the prevailing odorant for eight months and I thus tried to ensure that all periods of juvenile migration and possibly olfactory imprinting were covered.
2. PST stage: Exposure was carried out for 30 days starting at first morphological signs of PST (scale loss, silvering, higher activity when feeding and approaching rearing tanks).

The same odorant concentration that was used during juvenile imprinting, was also used when adult fish were tested for behavioural responses to the imprinting odorants. During juvenile imprinting exposure, odorants were administered by a constant drip from 25 L carboys, adjusted by precision dripping valves. The odorant was mixed into 22 L of well water and ran at a drip rate of 5 mL • min^{-1}. This way I composed a mixture of the well water specific background odorants and the potential imprinting odorant at a higher concentration. During the imprinting odorant exposure period, the rate of drip was controlled and adjusted twice daily. Identical carboys, with untreated water, were positioned on the rim of control group tanks to standardize between treatment and control groups. Odorant concentrations in the odorant tanks were determined according to the

flow rate through the fish rearing tanks. Substances and their respective concentrations used as imprinting odorants in this study are summarized in Table 2.1.

A minimum of 100 fish per treatment group were exposed to an odorant and/or a hormone. For experiments in adult fish, 5000 sockeye salmon were reared and remained untreated.

Table 2.2.: Timing of hormone treatments that correspond with $MnCl_2$ exposure at juvenile stages and with behavioural testing with $MnCl_2$ at age three (for explanation of life stage names see Figure 1.1).

Stage	Hormone and odorant exposure
fry	**T_3, T_4 and T_3+T_4** in feed for 30 days + $MnCl_2$, starting six weeks after first feeding
post PST	**T_3, T_4 and T_3+T_4** in feed for 30 days + $MnCl_2$, starting 90 days after first morphological signs of PST
two years of rearing without hormone or odorant exposure	
age three immature	**GnRH** 2 injections, 36h and 12h before testing into epaxial musculature of immature fish

Odorant exposure of juvenile sockeye salmon in combination with hormonal treatment

The timing of juvenile exposure to $MnCl_2$ in combination with both forms of thyroid hormones is summarized in Table 2.2.

Rearing between odorant and hormone exposure and behavioural testing of adult fish

Subsequent to the last odorant and/or hormone treatment (September in the year of first feeding) of juvenile fish, all sockeye were transferred into tanks with a diameter of 0.9 m (10 L • min^{-1} flow through tanks, water temperature 8.5 ± 0.5°C, aerated well water). Fish that did not show any signs of maturation 15 months after first feeding were transferred into tanks with a diameter of 1.8 m (25 L • min^{-1} flow through tanks, water temperature 8.5 ± 0.5°C, aerated well water) for rearing during year three and four.

2. Materials and methods for the experiments in this chapter only:

Odorant recognition testing in the behavioural trough and the y-maze waterfall

The behavioural trough and the Y-maze waterfall were used to assess behavioural responses to potential imprinting odorants 22 to 25 months after the exposure to the odorants and hormones (treatment groups) or the background water (naïve control fish). Fish from the same treatment group were tested in both experimental set-ups whereas the same individuals were tested in only one of the two set-ups. All treatment groups were labeled by the treatment that they experienced as juveniles.

Behavioural trough

The behavioural trough (length: 4.8 m, width: 1 m, height 0.5 m) was divided into ten inflow chambers, an undivided fish chamber and ten outflow chambers (Figure 2.1).

Water entered the trough through the inflow chambers and flowed through a perforated sheet of PVC (2 mm hole size, flow laminarizer) into the fish chamber (10 L • min^{-1} per chamber). A second perforated PVC divider allowed the water to leave the trough through one of the ten outflow chambers. Flow through each of the inflow and outflow compartments could be regulated and treated with an odorant.

Behavioural trough, experiment 1: Spatial profile of the odorant plume in the behavioural trough

Dye tests visualized the flow of the test odorants and demonstrated that the mixing occurred mainly on the margins of the plume and did not continue beyond the adjacent compartment on either side. Thus it was ensured that odorant plumes were spatially defined. Small turbulences, possibly created by the fish, were mimicked by hand movement and showed that the plume stayed stable and that fractions of the odorant were not distributed throughout the trough.

Behavioural trough, experiment 2: Tracking the swimming pattern of test fish in the behavioural trough (Figure 2.1)

Fish movement was recorded by using a tracking program designed for this project. The fish's x-y coordinates were recorded (represented by the cursor within an image of the behavioural trough on the computer screen) every 0.5 seconds for the duration of the observation. Fish movement could then be played back for visual and statistical analysis. The trough image on the computer screen was projected onto an observation mirror and superimposed on the reflection of the actual trough. This allowed

the observer to maintain continuous visual contact with the superimposed image of the fish and the cursor. The trough was illuminated with fluorescent bulbs along its whole length to ensure even lighting conditions throughout. Access to the ends of the trough was cut off by plastic netting material to avoid a corner hiding behaviour, observed when access to the corners was allowed in first experiments. Therefore, only compartment two to nine were accessible to the fish. Since fish had to slow down before they turn around in the corners, corners are automatically over represented when recording the frequency of stay in each compartment and hence compartment two and nine were never treated with odorants and were not considered when analyzing the swimming pattern statistically.

To start a behavioural trial, fish were caught with a dip net and transferred into a bucket under water. Then the water-filled bucket, including the fish, was quickly transported into the behavioural trough, where the fish was released under water. Since air exposure was avoided, stress was minimized and fish would acclimatize quicker. Fish behaviour was subsequently monitored every ten minutes, until a regular swimming pattern from end to end of the trough was observed. Fish that did not establish such a swimming pattern within three hours were excluded and transferred back into a holding tank (≈ 5%). Swimming pattern was then recorded for twenty minutes without odorant and for twenty minutes with the odorant running. Duration of stay and number of turns in each compartment were monitored and analyzed comparatively. Since the experimental design allowed for comparison of behaviour for the same individuals before and during exposure to odorants, the paired-t test was used to compare mean of duration and mean number of turns within the odorant compartment. To avoid inadvertently re-testing the same fish, all fish were transferred to separate holding tanks after testing.

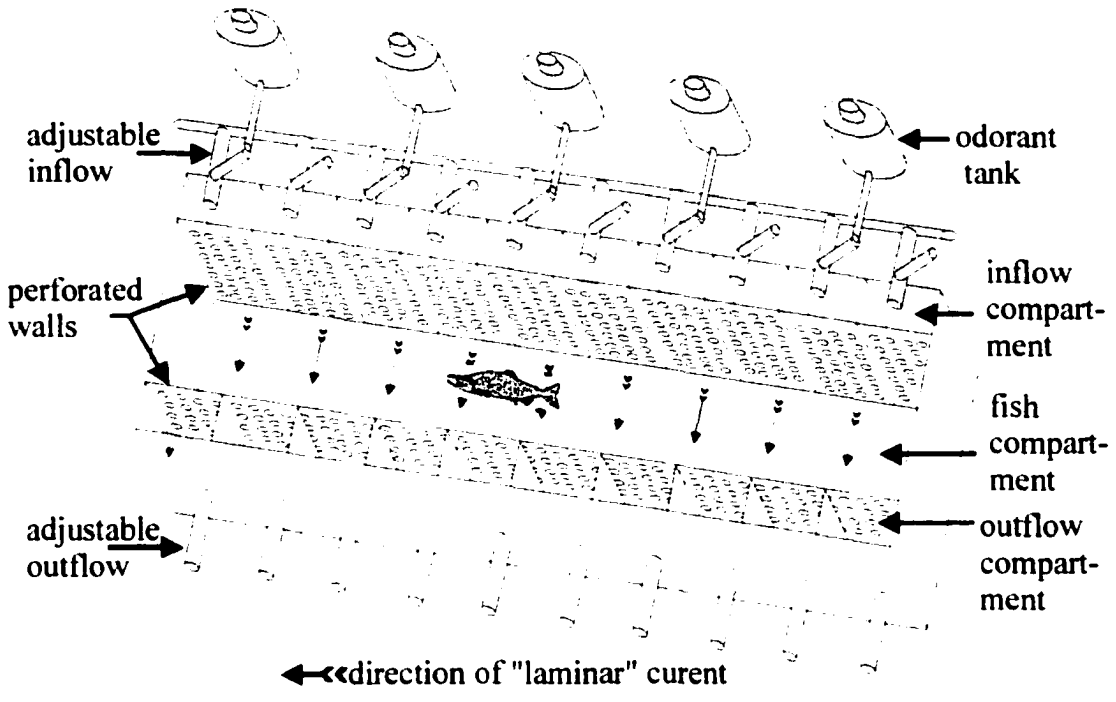


Figure 2.1.: The behavioural trough (adapted from Jones & Hara, 1985). Two perforated plates divide the trough interior into three longitudinal sections, an influent side, an undivided central channel in which a fish may swim freely, and an effluent side. The length of the fish section is marked with numbers one to ten. The influent side is partitioned into ten cells, each receiving its own adjustable water supply, which can be scented by dropping an odorant out of an odorant tank. Incoming water mixes in the cells, passes through the first divider, flows across the central fish channel, moves through the second divider into the effluent side, and finally exits by one of the ten outflows. Fish swim perpendicular to the current and can scan the different water masses for olfactory stimuli. Dimensions: length 4.8 m, width 1 m (inflow and outflow cells 0.2 m respectively, fish channel 0.6 m), height 0.5 m.

The waterfall set-up

Waterfall set-up, experiment 1: Influence of flow, maturity and gender on the behavioural performance

Fish tested in the waterfall were three years of age, immature and had never been exposed to artificially enhanced concentrations of imprinting odorants. The waterfall consisted of two chambers (2.4 m long, 0.6 m wide and 1 m deep) with underwater observation windows that were connected via a waterfall with a drop of 20 cm. A discharge of 200 L per minute of de-chlorinated water at a temperature of 15 °C was flowing through the chambers and over the waterfall. Individual test fish were transferred in a water bucket from their holding tanks into the lower chamber of the simple waterfall set-up. The behavioural response of 20 individual fish was monitored for 2 h each. After the first round of testing all 20 fish were transferred back into holding tanks. Ten or half of the fish were subsequently sedated and injected with 20 $\mu g \bullet kg^{-1}$ body weight of GnRH analogue (D-Ala-6, des Gly^{10} ethyl amide, dissolved in a 9‰ saline) while the other ten fish were injected with a saline solution (controls). The following day the injection procedure was repeated to stimulate pituitary GTH synthesis and secretion respectively (N. Sherwood, personal communication). Three days after the first test, sockeye salmon were re-tested in the simple waterfall set-up and behavioural performance was compared between test one and test two. If GnRH did not affect the behaviour of salmon in response to a waterfall, it was expected that: the behavioural performance in test number two should not differ between GnRH and saline injected fish and that the behavioural differences between test number one and test number two should

be consistent over both treatment groups. As a measure of acclimatization to the waterfall set-up, I recorded the duration of the motionless period that usually occurs after fish have been transferred to a new environment. Number of passes trough the waterfall and the number of jumps into the waterfall were recorded to determine general activity and motivation to overcome a waterfall.

Waterfall set-up, experiment 2: Odorant recognition in the y-maze waterfall

The main structure of the Y-maze waterfall (Figure 2.2.) is a concrete raceway with a length of 15m and a width of 2.4 m. The upper part of the raceway was divided into two spawning compartments of 4m length and equal width of 1.2 m. A constant flow of 200 $l \bullet min^{-1}$ was presented in both of the spawning compartments (10 cm layer of gravel on the bottom) and spilled over a 15 cm high and 40 cm wide waterfall into the main undivided swimming chamber. In the swimming chamber (8 m long, 2.4 m wide and 0.3 m deep), fish could move freely and access the two waterfalls.

In preparation for an experiment, test groups were left at the outflow end of the concrete raceway one night prior to experiments for acclimatization. The rearing area (3 m length, 2.4 m width) was divided from the swimming chamber by black tarp attached to a supporting metal frame to avoid visual contact of the test fish and the fish that still had to be tested. Test fish were transferred from the rearing area into the swimming chamber with a dip net. Since individuals only had to be lifted over the partitioning plastic tarp, stressful air exposure was minimized. Positioned on the inflow end of both spawning chambers were 25 L carboys that contained either straight water or a water odorant mixture.

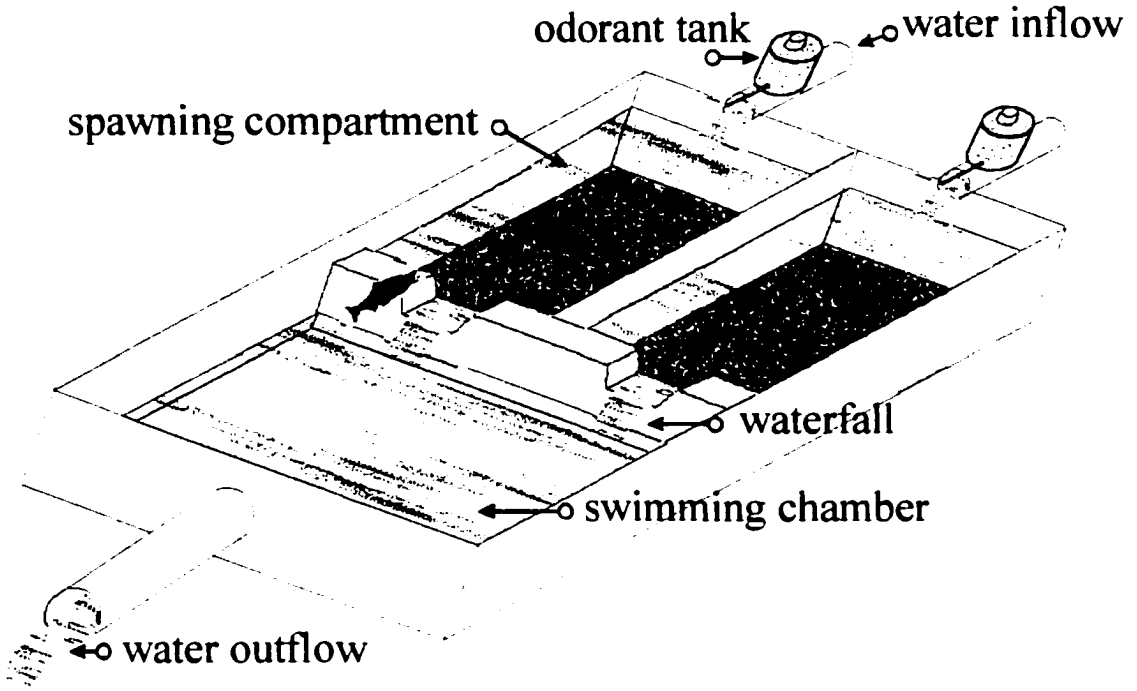


Figure 2.2.: The Y-maze waterfall is divided into two spawning compartments (10 cm layer of gravel on the bottom) that are 4 m long and 1.2 m wide and the undivided swimming chamber (8 m long, 2.4 m wide and 0.3 m deep) where fish can move freely and choose to jump up one of the two waterfalls. Both spawning chambers and therefore both of the waterfalls could be treated with odorants by regulated discharge of odorants from the odorant tanks.

The carboy discharge rate could be regulated by valves to adjust odorant concentration to the water flow. The compartment with odorant treatment was fixed within each day of testing but randomly chosen from day to day (well water dripped into the other compartment). The odorant drip was stopped over night to allow for rinsing of the whole raceway and to avoid odorant residue build-up in either one of the spawning chambers. Fish behaviour in the swimming chamber was observed through a small slit in black plastic tarp that was attached to wooden support frames, which served as an experimenter

blind. A test fish was scored as soon as it jumped up the waterfall and entered the spawning chamber. fish that had not jumped up either waterfall after 4 h were scored as "non-jumping" fish. The ratio between jumping and non-jumping fish was used to determine the motivation to overcome the waterfall. Experimental trials were followed by 30 min pauses to ensure that any odorant trails emitted from the previously tested fish would be highly diluted.

II. 3. Results

1.Outcome of testing in the behavioural trough:

1.a. Behavioural responses of naïve[1] fish to potential imprinting odorants:

Manganese Chloride ($MnCl_2$)

$MnCl_2$ was found at a concentration of $2 \bullet 10^{-7}$ M in Fulton River (water analysis carried out in 1998 by DFO Canada, Table 2.3), one of the natal streams for the tested sockeye salmon. When tested for changes in swimming pattern in the behavioural trough (Figure 2.3.a. & b.) neither three year old immature ($\alpha = 0.988$) nor mature ($\alpha = 0.502$) fish showed a significant change in the percentage of time that the fish stayed in the $MnCl_2$ (10^{-6} M) treated compartments. $MnCl_2$ therefore neither repels nor attracts sockeye salmon that have not been previously exposed to it.

Table 2.3.: Water Analysis: Fulton River, tributary of Babine Lake, northern British Columbia Canada, Carried out in June 16th, 1998.

Metal	Result [mg · L^{-1}]	Molar concentration [M]
Barium	0.02	$1 \cdot 10^{-7}$
Calcium	10	$2.5 \cdot 10^{-5}$
Iron	0.16	$2.8 \cdot 10^{-6}$
Magnesium	2.3	$1 \cdot 10^{-4}$
Manganese	0.012	$2 \cdot 10^{-7}$
Silicon	2.18	$7.8 \cdot 10^{-5}$
Strontium	0.065	$7 \cdot 10^{-7}$
Zinc	0.007	$1 \cdot 10^{-7}$

[1] Naïve fish are defined as fish that have never been artificially exposed to an odorant or a hormone prior to testing for odorant recognition

Figure 2.3.a.

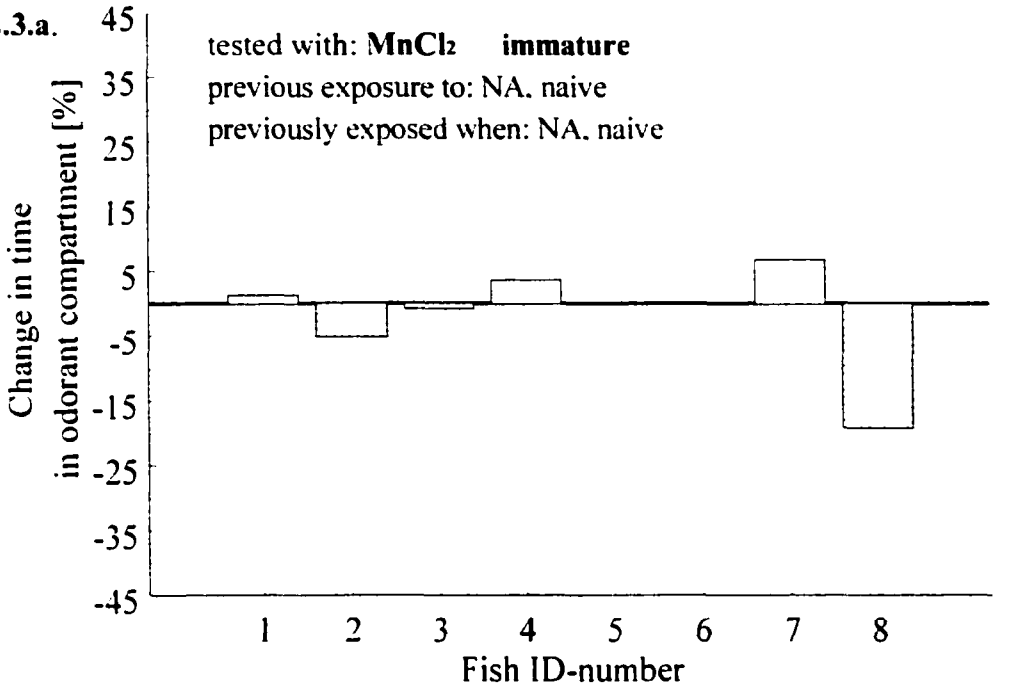


Figure 2.3.b.

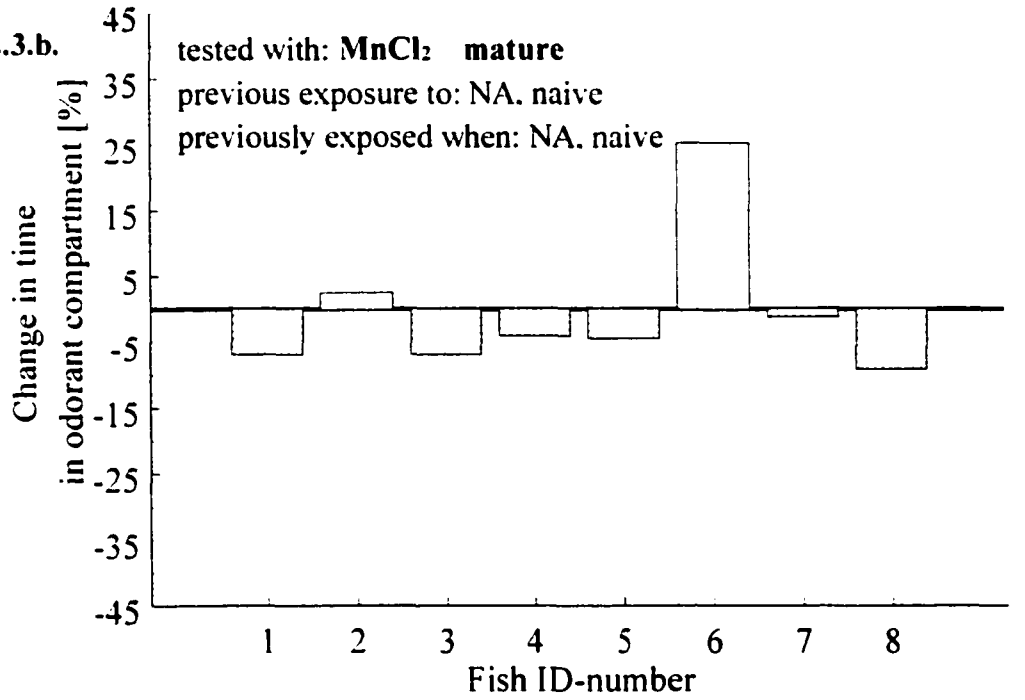


Figure 2.3.: Changes in time in the $MnCl_2$ (10^{-6} M) treated area of the behavioural trough (NA = fish from this cohort were never artificially exposed to an odorant or hormone). Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of stay in the odorant treated area. Ten three-year-old immature (2.3.a.) and nine three-year-old mature (2.3.b.) fish were tested. Frequency of time in the odorant treated area did not change in response to $MnCl_2$ in immature (paired t-test, $p = 0.565$) or mature (paired t-test, $p = 0.876$) sockeye salmon.

Cholic acid

Immature sockeye salmon (Figure 2.4.a.) did not exhibit a significant ($\alpha = 0.148$) behavioural responses to the presence of cholic acid at a concentration of 10^{-8} M. In contrast, mature sockeye salmon (Figure 2.4.b.) avoided the odorant compartment ($p = 0.012$) once cholic acid was present at a concentration of 10^{-8} M. In summary, three-year-old sockeye were repelled by cholic acid once they reached maturity but not at immaturity.

Cupric chloride ($CuCl_2$)

Immature (Figure 2.5.a.) and mature sockeye (Figure 2.5.b.) were strongly repelled by $CuCl_2$ at a concentration of 10^{-6} M. All of the mature ($p = 0.008$), and seven out of eight immature ($p = 0.001$) sockeye avoided the odorant compartment when $CuCl_2$ was present.

L-glutamine

The presence of L-glutamine (10^{-5} M) did not change the time that sockeye salmon stayed in the odorant treated compartments significantly (eight immature fish, Figure 2.6.a., $p = 0.569$; 12 mature fish, Figure 2.6.b., $p = 0.192$).

Barium Chloride ($BaCl_2$)

Just like $MnCl_2$, $BaCl_2$ was found at concentrations around $1 \cdot 10^{-7}$ M in Fulton River (Table 2.3), one of the natal streams for the tested sockeye salmon. In the behavioural trough, eight immature (Figure 2.7.a.) and 16 mature (Figure 2.7.b.) sockeye salmon did not significantly alter their frequency of stay in the odorant compartment in response to the presence of $BaCl_2$ (immature $p = 0.454$, mature $p = 0.993$).

Figure 2.4.a.

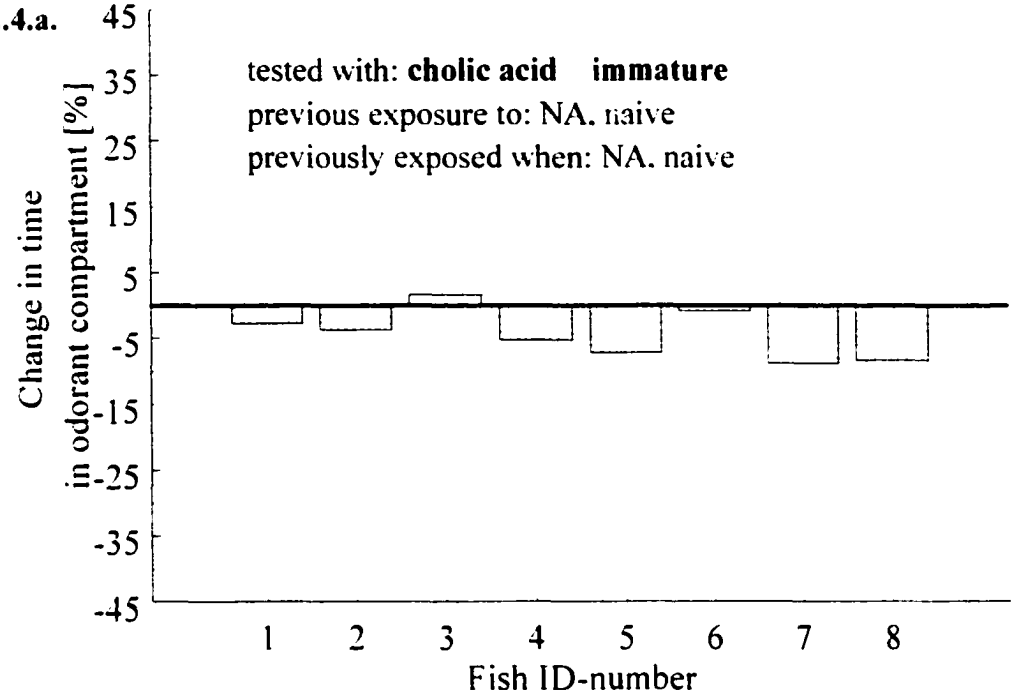


Figure 2.4.b.

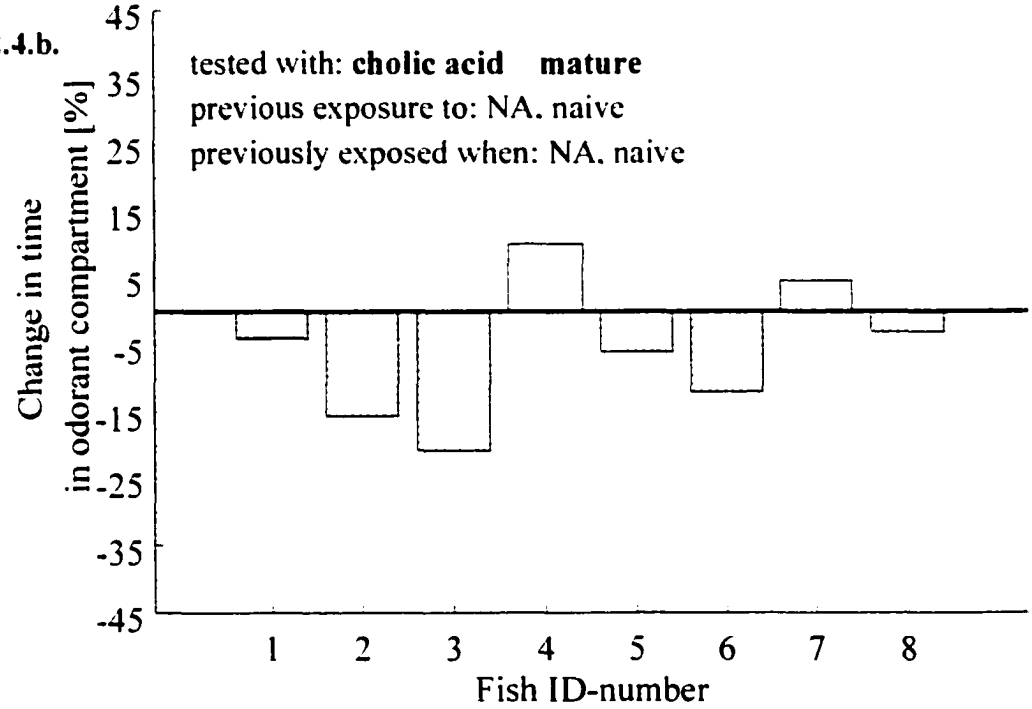


Figure 2.4.: Changes in time in the cholic acid (10^{-8}M) treated area of the behavioural trough. Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of stay in the odorant treated area. Eight three-year-old immature (2.4.a.) and eight three-year-old mature (2.4.b.) fish were tested. Frequency of time in the odorant treated area in response to cholic acid did not change in immature (paired t-test, $p = 0.148$) but decreased significantly in mature (paired t-test, $p = 0.012$) sockeye salmon.

Figure 2.5.a.

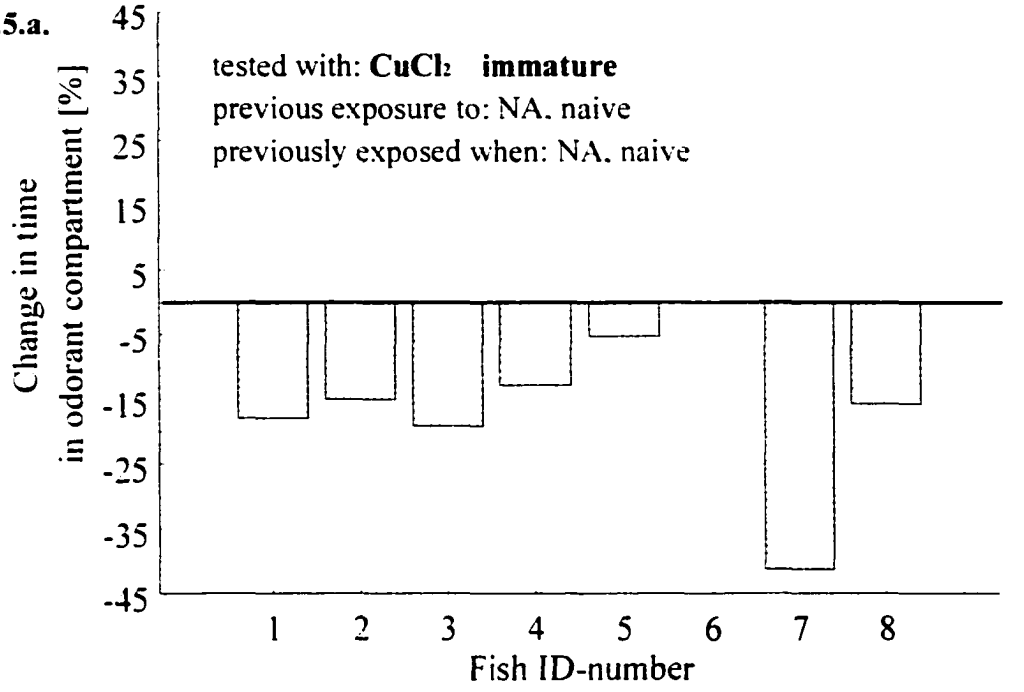


Figure 2.5.b.

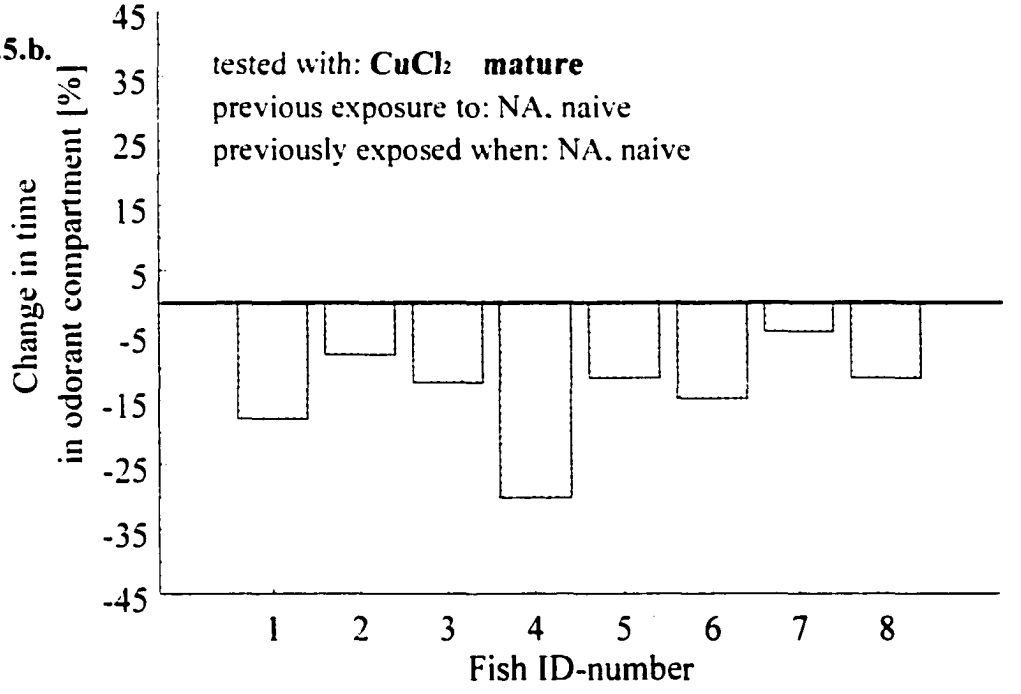


Figure 2.5.: Changes in time in the cupric chloride ($CuCl_2$ at 10^{-6}M) treated area of the behavioural trough. Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of stay in the odorant treated area. Eight three-year-old immature (2.5.a.) and eight three-year-old mature (2.5.b.) fish were tested. Frequency of time in the odorant treated area decreased highly significant in response to $CuCl_2$ in immature (paired t-test, $p = 0.008$) and in mature (paired t-test, $p = 0.001$) sockeye salmon.

Figure 2.6.a.

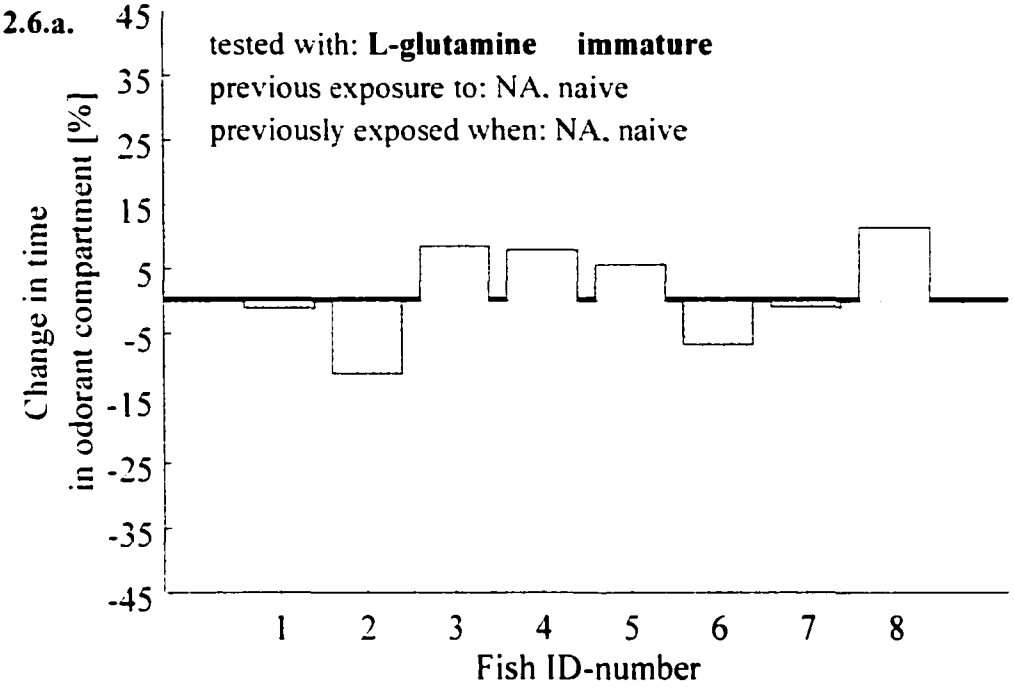


Figure 2.6.b.

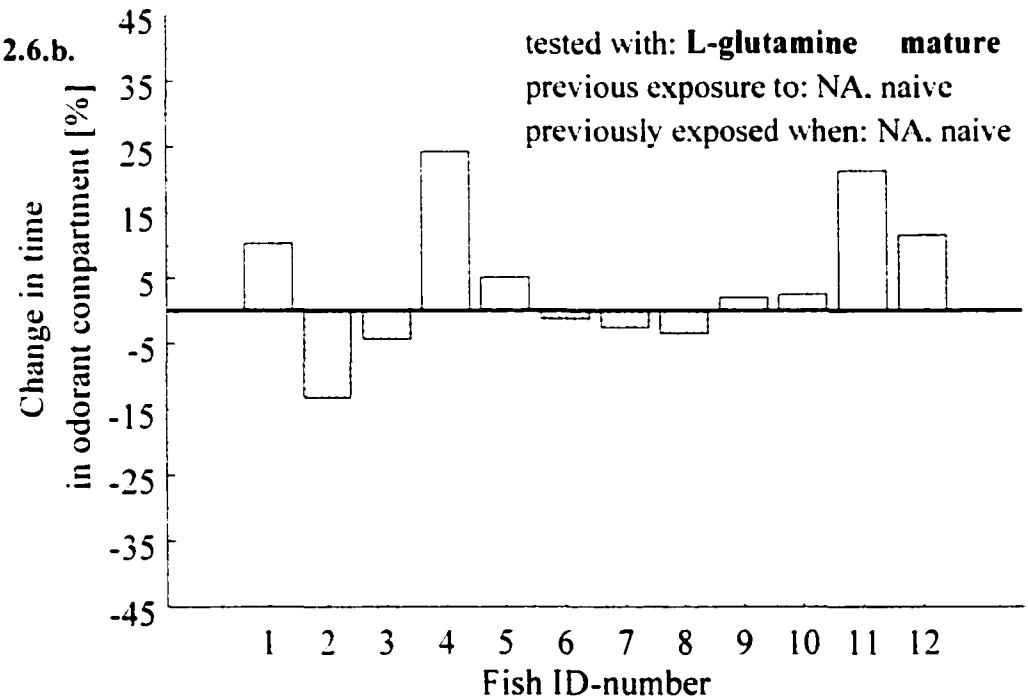


Figure 2.6.: Changes in time in the L-glutamine treated (10^{-5} M) area of the behavioural trough. Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of stay in the odorant treated area. Eight three-year-old immature (2.6.a.) and twelve three-year-old mature (2.6.b.) fish were tested. Frequency of time in odorant compartments did not change significantly in response to L-glutamine in immature (paired t-test, $p = 0.569$) or in mature (paired t-test, $p = 0.192$) sockeye.

Figure 2.7.a.

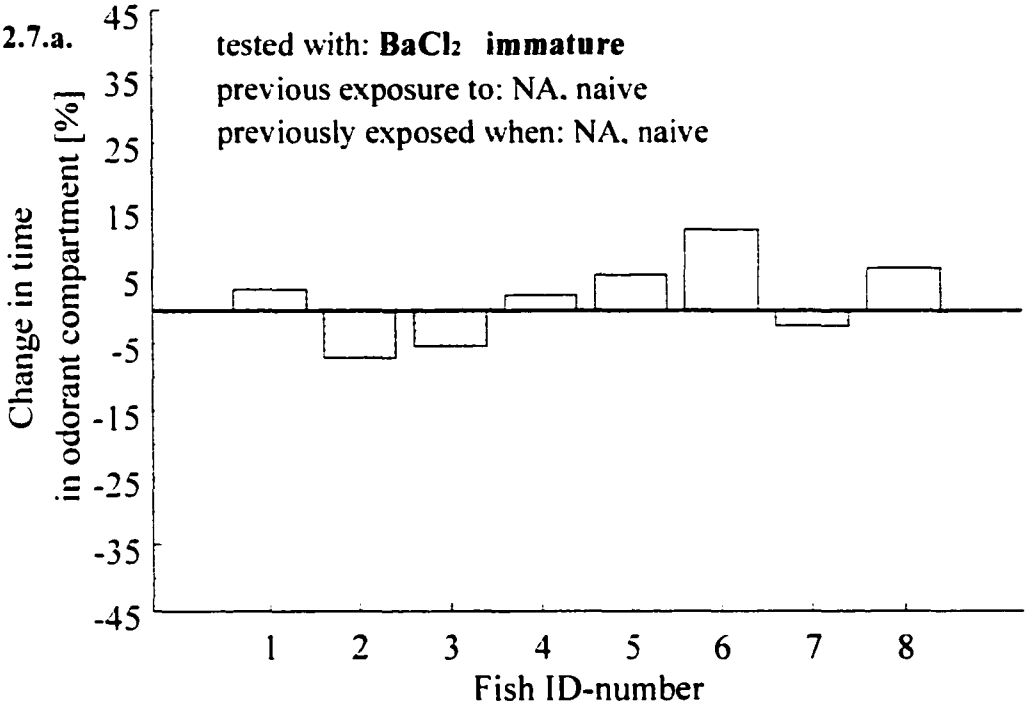


Figure 2.7.b.

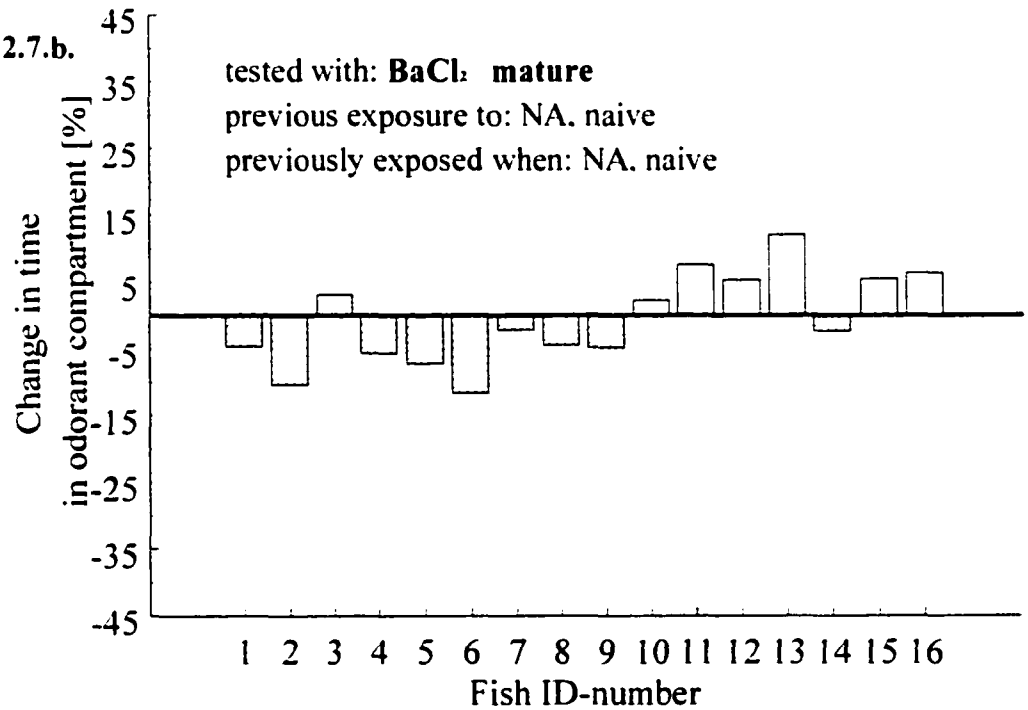


Figure 2.7.: Changes in time in the barium chloride ($BaCl_2$ at 10^{-6} M) treated area of the behavioural trough. Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of stay in the odorant treated area. Eight three-year-old immature (2.7.a.) and 16 three-year-old mature (2.7.b.) fish were tested. Frequency of time in the odorant compartments did not change significantly in response to $BaCl_2$ in immature (paired t-test, $p = 0.454$) or in mature (paired t-test, $p = 0.993$) sockeye.

1.b. Behavioural response of three-year-old fish that were exposed to the testing odorants at juvenile stages

Three-year-old sockeye salmon that had experienced juvenile exposure to $MnCl_2$, $BaCl_2$ or L-glutamine without thyroid hormone treatment displayed no significant change in their swimming pattern when presented with either one of the odorants (Table 2.3.).

1.c. Behavioural response of three-year-old fish that were exposed to the testing odorants and thyroid hormones at juvenile stages

Control groups: Juvenile exposure to $MnCl_2$

Since thyroid hormones were dissolved in ethanol, control groups were fed with ethanol treated feed without thyroid hormone addition while being exposed to $MnCl_2$. Neither immature nor mature fish out of the control groups were attracted or repelled when tested in the behavioural trough at age three (Table 2.4.).

Hormone groups: Simultaneous juvenile exposure to $MnCl_2$ and T_3 or T_4 independently or T_3T_4 combined

Groups that were treated with T_3 or T_4 while they were exposed to $MnCl_2$ 42 days after first feeding or 90 days after the first morphological signs of PST did not react significantly to $MnCl_2$ two years later in the behavioural trough (Table 2.4.). This lack of a behavioural response was consistent between three-year-old immature and mature fish. However, a combined juvenile T_3T_4 treatment concurrent with exposure to $MnCl_2$ significantly changed the behavioural reaction of three-year-old sockeye salmon to the imprinting odorant regardless of the timing of juvenile odorant exposure (Figure 2.8.).

Table 2.3.: Summary table for behavioural responses of three-year-old sockeye that were exposed to odorants but not to hormones during juvenile odorant exposure. The table shows testing odorants at age three, physiological state, developmental stage during juvenile odorant exposure, number of fish tested (N), p-value for the paired t-test and mean value of the percentage that the fish stayed in the odorant compartments without odorant and while the odorant was running ± the standard error of the prevailing mean.

Testing odorant	Age	Physiological state	Odorant of juvenile exposure	N	p-value, paired t-test	Mean no odorant ± SE	Mean odorant ± SE
$MnCl_2$	3	immature	$MnCl_2$ during PST	8	0.870	23.1 ± 6.9	22.7 ± 8.7
$MnCl_2$	3	mature	$MnCl_2$ during PST	10	0.557	25.8 ± 1.9	28.8 ± 4
$BaCl_2$	3	immature	$BaCl_2$ during PST	9	0.077	27.6 ± 1.7	23 ± 2.4
$BaCl_2$	3	mature	$BaCl_2$ during PST	9	0.652	25.7 ± 2.9	23.9 ± 3.4
L-glutamine	3	immature	L-glutamine during PST	10	0.852	29.9 ± 2.5	30.1 ± 3.4
L-glutamine	3	mature	L-glutamine during PST	8	0.389	30.2 ± 3	31.4 ± 3.9
L-glutamine	3	immature	L-glutamine first feeding until 3 months after PST	7	0.368	27.9 ± 1.1	25.2 ± 2.8
L-glutamine	3	mature	L-glutamine first feeding until 3 months after PST	9	0.261	27.8 ± 1.8	25.9 ± 2.9

Table 2.4.: Summary table for behavioural reactions of three-year-old sockeye salmon that were exposed to $MnCl_2$ (10^{-6} M) with or without simultaneous exposure to T_3 or T_4 independently. The table shows maturity state, odorant and hormone that fish were exposed to, start and length of exposure period, number of fish tested (N), p-value for the paired t-test and mean value of the percentage that the fish stayed in the odorant compartments without odorant and while the odorant was running ± the standard error of the prevailing mean. Since the p-value determined in the paired t-test was greater than 0.05 for all groups, no significant behavioural reaction to $MnCl_2$ could be observed.

Subspecies	Mature	Odorant + hormone exposed to as juveniles	Start of exposure	N	α (paired t-test)	Mean no odorant ± SE	Mean odorant ± SE
Sock	no, age 3	$MnCl_2$, no hormone, feed ethanol treated	42 days after first feeding for 30 days	8	0.331	27.9 ± 5.2	25.6 ± 4.4
Kok	yes, age 3	$MnCl_2$, no hormone, feed ethanol treated	42 days after first feeding for 30 days	9	0.517	34 ± 2.7	31.3 ± 4.7
Sock	no, age 3	$MnCl_2$, no hormone, feed ethanol treated	90 days after first morphological signs of PST	10	0.510	32.7 ± 8.2	29.4 ± 5.9
Sock	yes, age 3	$MnCl_2$, no hormone, feed ethanol treated	90 days after first morphological signs of PST	10	0.222	26.7 ± 4.3	23.7 ± 4.4
Kok	yes, age 3	$MnCl_2$, T_3 for 30 days	42 days after first feeding	8	0.847	26 ± 2.2	26.7 ±2.5
Kok	yes, age 3	$MnCl_2$, T_4 for 30 days	42 days after first feeding	10	0.830	22.8 ± 0.8	22.4 ± 2
Sock	yes, age 3	$MnCl_2$, T_3 for 30 days	90 days after first morphological signs of PST	9	0.094	22.9 ± 1.8	28.1 ± 1.9
Sock	yes, age 3	$MnCl_2$, T_4 for 30 days	90 days after first morphological signs of PST	8	0.844	24.6 ± 3.8	23.5 ± 3.8
Kok	no, age 3	$MnCl_2$, T_4 for 30 days	42 days after first feeding	8	0.197	28.8 ± 1.4	25.5 ± 1.7
Sock	no, age 3	$MnCl_2$, T_4 for 30 days	90 days after first morphological signs of PST	9	0.087	29.7 ± 2.6	25.8 ± 2.3

Figure 2.8.a.

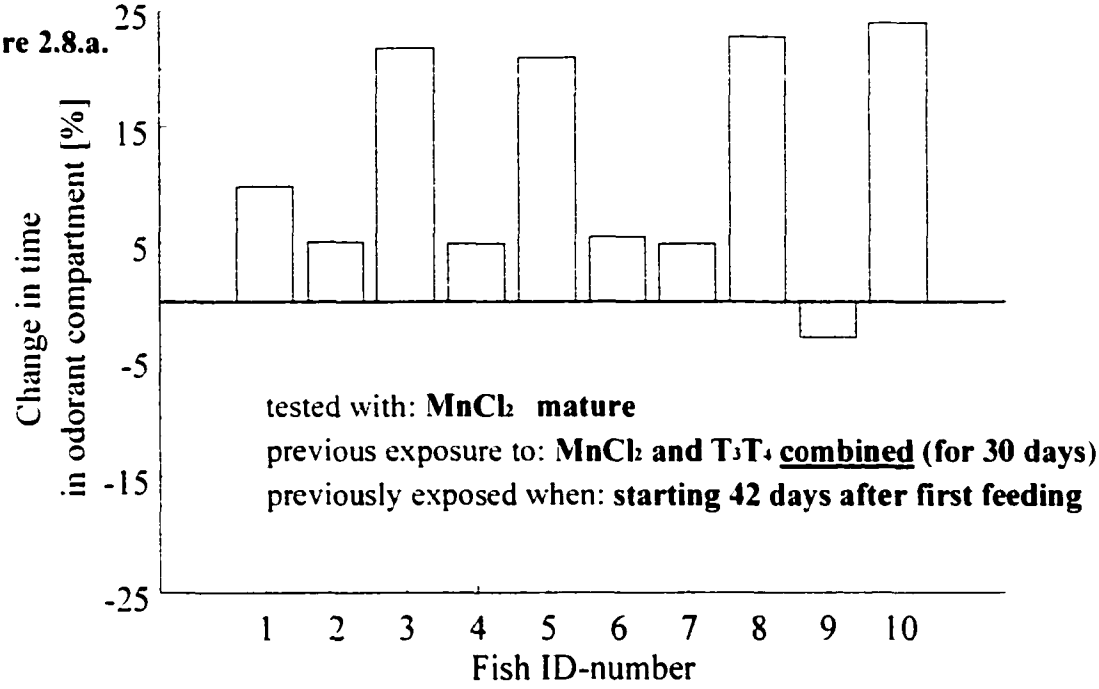


Figure 2.8.b.

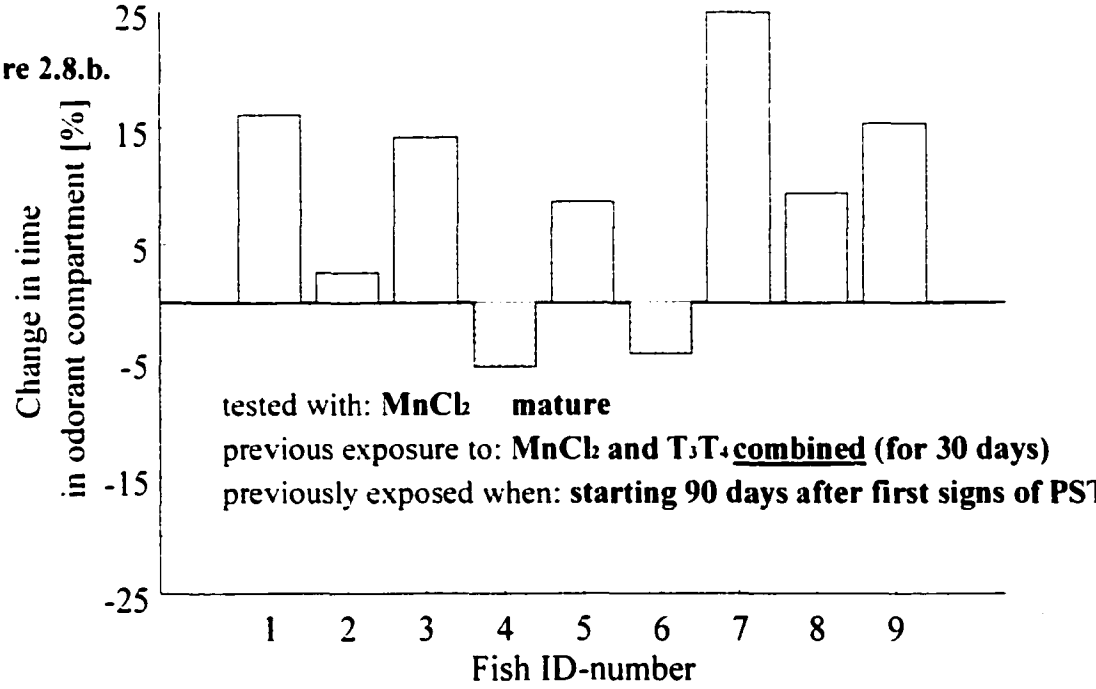


Figure 2.8. legend: Changes in time stayed in the $MnCl_2$ (10^{-6} M) treated area of the behavioural trough. Negative values (striped bars) represent a decreased and positive values (clear bars) represent an increased frequency of residence in the odorant treated area. I tested ten three-year-old mature (2.8.a.) sockeye that were exposed to $MnCl_2$ and T_3T_4 combined, 42 days after first feeding (Figure 2.8.a.) (paired t-test, $p = 0.004$) and nine three-year-old mature sockeye that were exposed 90 days after first morphological signs of PST (Figure 2.8.b.) (paired t-test, $p = 0.027$). Both treatment groups were significantly attracted to $MnCl_2$.

Three-year-old mature sockeye salmon that were exposed to $MnCl_2$ and T_3T_4, 42 days after first feeding (Figure 2.8.a.) or 90 days after the onset of PST (Figure 2.8.b.), resided significantly more in the odorant compartments when the imprinting odorant was running ($p = 0.004$ and $p= 0.027$ respectively). Increased T_3T_4 plasma levels concurrent with exposure to an odorant, $MnCl_2$ in this study, within the first year of ontogeny seemed to be the basis for olfactory imprinting.

2. Results from the Y-maze waterfall

2.a. Determination of general motivational factors to overcome a waterfall

The amount of water spilling over the waterfall was a crucial in motivating two-year-old immature sockeye salmon to overcome the fall (Figure 2.9.). At four different flows (50, 150, 250 and 350 L • min^{-1}) the percentage of fish (eight per group) that overcame the fall ranged from one out of eight fish (12.5 %) at 50 and 150 L • min^{-1} to five out of eight fish (62.5 %) at a flow of 250 L • min^{-1}. At 350 L • min^{-1}, four out of eight or 50% of the tested fish overcame the waterfall.

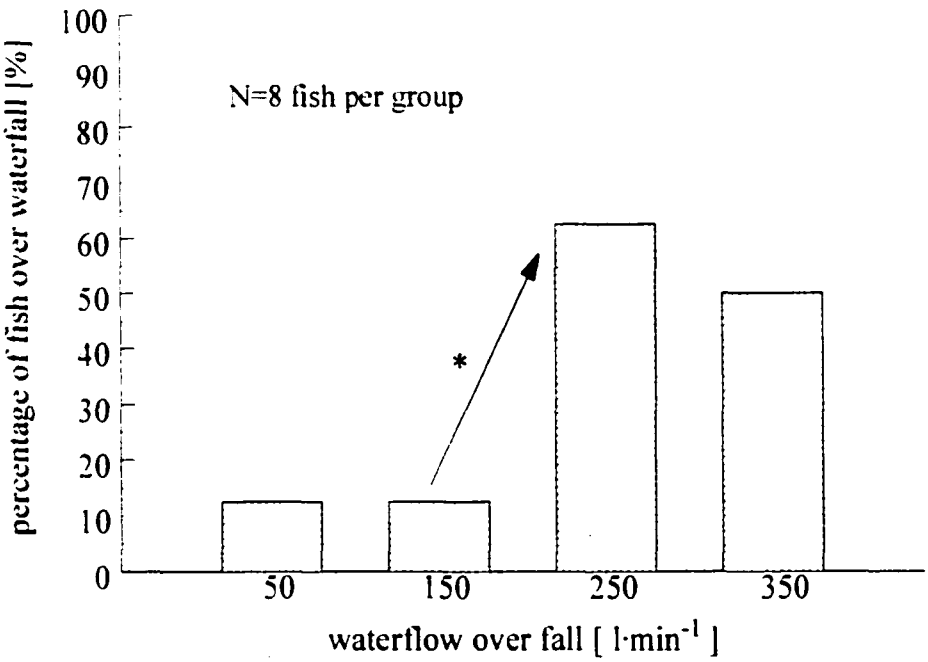


Figure 2.9. (legend): The influence of flow on the percentage of fish that overcame the waterfall in the y-maze waterfall experiment. At each of the four flow regimes (50, 150, 250 and 350 L • min^{-1}) we tested eight two-year-old immature sockeye salmon. Fish were scored as soon as they overcame the fall for the first time. Flows of 50 or 150 L • min^{-1} motivated only one out of eight fish (12.5 %) to jump. At a flow of 250 and 350 L • min^{-1} fish were significantly (Chi-Square test, $\alpha = 0.039$) more motivated and the percentage increased to five out of eight (62.5 %) and four out of eight (50 %) jumping fish respectively. (The Chi-Square test is used to compare an observed with an expected frequency, whereas the expected frequency in this case, was the jumping frequency of the fish at the lowest water discharge of 50 L • min^{-1}).

Figure 2.10.a.

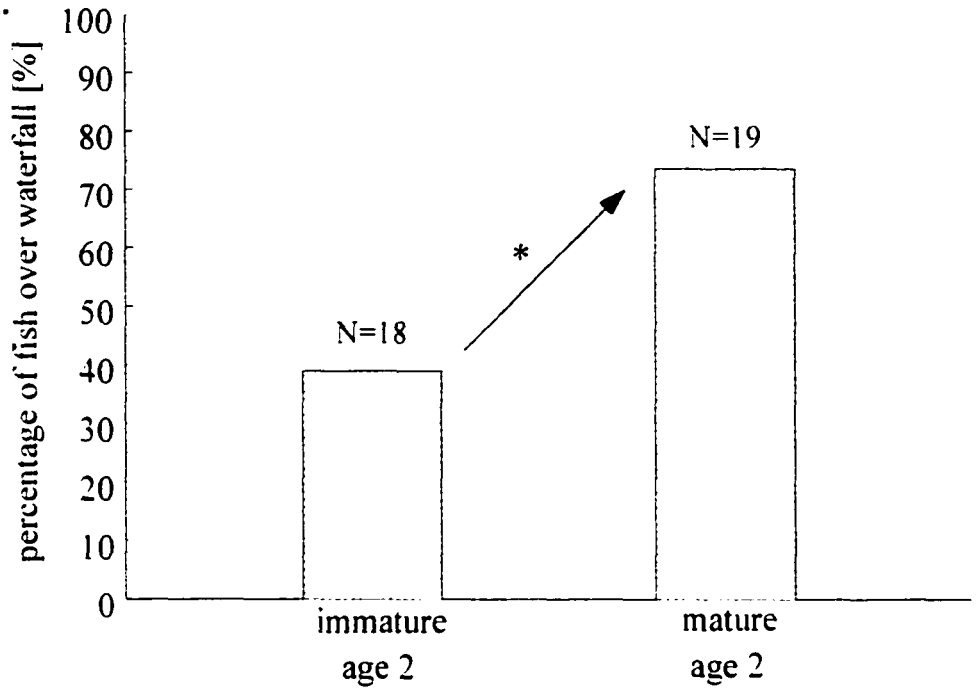


Figure 2.10.b.

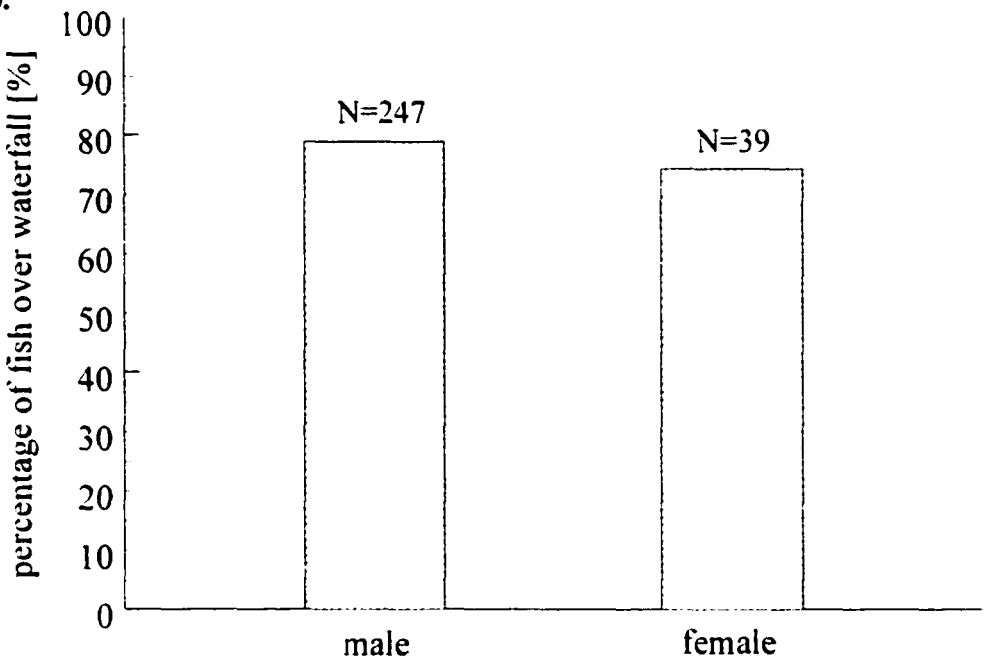


Figure 2.10. a. and b. (legend next page)

Figure 2.10. a. and b: Figure 2.10.a. shows the correlation between the motivation to overcome a waterfall and the state of maturity. Seven out of 18 (38.8 %) immature and 14 out of 19 (73.7 %) mature sockeye salmon jumped up one of the waterfalls in the Y-maze waterfall set-up. The difference in percentage between immature and mature fish is significant (Chi-Square test, $p = 0.033$).
In Figure 2.10.b. the percentage of mature sockeye that overcame a waterfall is plotted against gender. No significant correlation between gender and waterfall jumping motivation could be observed (Chi-Square test, $p = 0.518$): 195 out of 247 or 78.9 % of the males and 29 out of 39 or 74.3 % of the females overcame the waterfall. Due to the fact that males mature in average one year before female sockeye salmon a higher number of mature males was available for testing among the three-year-old sockeye at the time of testing, in the fall of 1997.

The difference in the number of fish that overcame the waterfall at flows ≤ 150 L • min^{-1} and ≥ 250 L • min^{-1} was significant (Chi Square Test, $p = 0.039$). The percentage of fish that overcame the waterfall at the lowest discharge rate of 50 L • min^{-1} was the expected frequency for the Chi-Square test that all other observed frequencies were tested against. In accordance with this finding the flow in all subsequent experiments in the Y-maze waterfall was kept at 200-250L • min^{-1} in each of the two waterfalls.

The state of maturity and its influence on the motivation of sockeye salmon to overcome a waterfall was the next factor that was investigated (Figure 2.10.a.). I tested 18 immature and 19 mature three-year-old sockeye salmon at a flow rate of 250 L • min^{-1} over both of the falls. Seven out of 18 (38.8 %) immature and 14 out of 19 (73.7 %) mature sockeye salmon overcame the falls (Figure 2.10.a.). This difference in frequency was significant determined by the Chi-Square test (expected frequency = 38.8 %, $p = 0.033$) and one can conclude that mature fish are more motivated to overcome a waterfall than immature fish.

Since it is possible to determine the gender of mature fish by visual examination of external morphological features, the influence of gender on the motivation to overcome a waterfall was determined in mature sockeye salmon. No significant difference (Chi-Square test, expected frequency = 78.9 %, $\alpha = 0.518$) to overcome a waterfall was observed between genders (Figure 2.10.b.) (195 out of 247 or 78.9 % males; 29 out of 39 or 74.3 % females).

2.b. Influence of GnRH on behaviour of sockeye salmon

To investigate possible GnRH effects, 20 immature sockeye salmon were tested twice in a waterfall set-up. In both tests, the time to the start of swimming behaviour, the number of passes through the plume of the waterfall and the number of jump attempts into the waterfall were recorded. Between first and second test, half of the fish were injected twice with a saline solution (controls) and the other half was injected twice with GnRH.

Time to move, increased significantly in control fish (paired t-test, $\alpha = 0.021$) from 4.9 ± 1.7 min in test one, to 24.9 ± 8.4 min in test two. In GnRH injected fish the time to move remained constant (paired t-test, $\alpha = 0.922$) (test one: 10 ± 2.1, test two: 9.3 ± 5.6) (Figure 2.11.a.).

Figure 2.11.a.

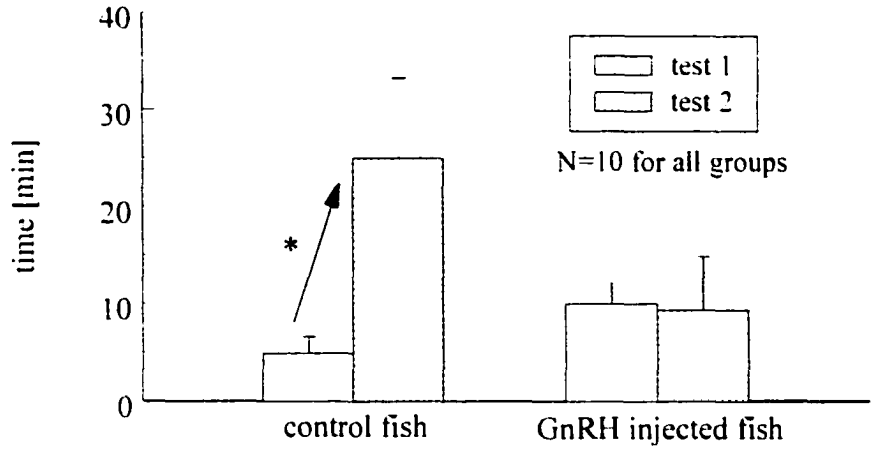


Figure 2.11.b.

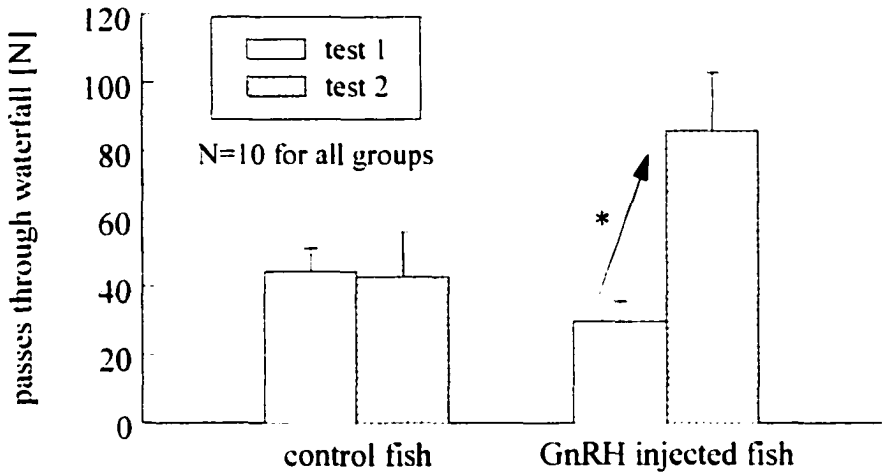


Figure 2.11.c.

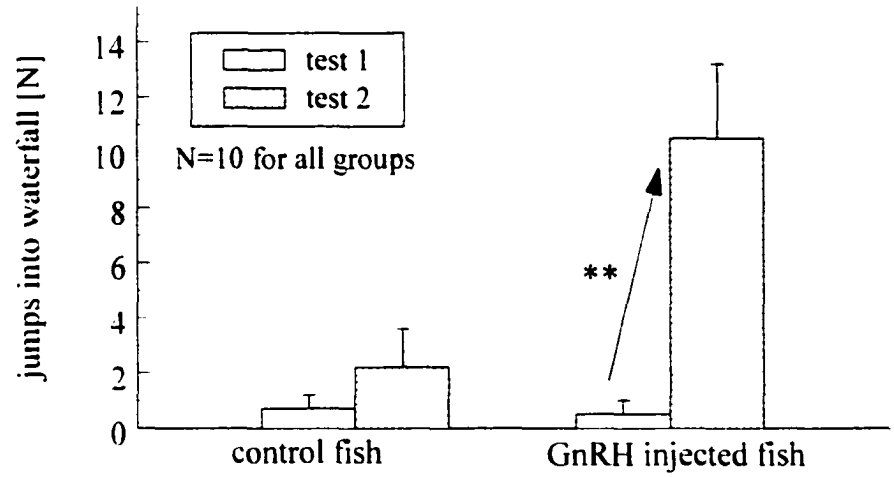


Figure 2.11. (legend next page)

Figure 2.11. a.+b.+c: Figures 2.11 a.,b. and c. describe GnRH caused changes in the behavioural reaction of sockeye salmon confronted with a waterfall. In test two half of the 20 fish, that were tested in test one, were injected with a saline solution (control fish) while the other half was injected with GnRH (GnRH injected fish). In Figure 2.11.a. I measured the period of time between transfer into the waterfall tank and start of swimming activity. Control fish started to swim after a significantly (paired t-test, α = 0.021) longer period of time in the second test (24.9 ± 8.4 min) when compared with test one (4.9 ± 1.7 min). In GnRH injected fish the time between tank transfer and start of swimming activity did not change significantly (paired t-test, α = 0.922) (test 1: 10 ± 2.1 min; test 2: 9.3 ± 5.6 min). The number of passes through the waterfall (Figure 2.11.b.) did not change significantly for control fish but increased significantly for GnRH injected sockeye salmon (control: paired t-test, α = 0.909, test one = 44.3 ± 7 passes, test two = 42.8 ± 13.2 passes; GnRH injected: paired t-test, α = 0.01, test one = 29.9 ± 5.8 passes, test two = 85.9 ± 16.9 passes). Jumping activity (Figure 2.11.c.), did not change significantly in control fish but increased in a highly significant manner for the GnRH injected sockeye (control: paired t-test, α = 0.296, test one = 0.7 ± 0.5 jumps, test two = 2.2 ± 1.4 jumps; GnRH injected: paired t-test, α = 0.007, test one = 0.5 ± 0.5 jumps, test two = 10.5 ± 2.7 jumps).

The number of passes through the waterfall (Figure 2.11.b.), interpreted as overall activity, increased significantly (paired t-test, α = 0.01) in the GnRH injected fish (test one: 29.9 ± 5.8 to test two: 85.9 ± 16.9) but stayed nearly unchanged for the control group (test 1: 44.3 ± 7 to test 2: 42.8 ± 13.2). Hence GnRH enhanced the overall activity of immature sockeye salmon when confronted with a waterfall.

Jumping activity (Figure 2.11.c.), did not change significantly in control fish (paired t-test, α = 0.296) in test two but increased in a highly significant manner for the GnRH injected sockeye (paired t-test, α = 0.007). GnRH therefore increased the motivation of sockeye salmon to jump into a waterfall in this study.

2.c. The behavioural response of immature and mature sockeye salmon to odorants in the y-maze waterfall set-up

In the preceding section, the influence of state of maturity, gender and GnRH on the behavioural response of sockeye salmon to a waterfall was determined. In the next experimental step, I added a second waterfall and an odorant that could be presented in one of the two waterfalls.

In Figure 2.12.a. the percentage of fish that overcame the waterfall is plotted against six different treatment groups. The expected value for the Chi-Square test, used for statistical analysis, was 71.4 % based on the fact that 15 out of 21 or 71.4 % of the control fish (no treatment) overcame the waterfall.

All groups were exposed to the odorant or odorant and hormone treatment for 30 days, starting 90 days after first morphological signs of smolting, two years prior to testing. Juvenile exposure to $MnCl_2$, $MnCl_2$ and T_3 and $MnCl_2$ and T_4 had no significant effect on the percentage of immature fish that mastered either one of the two waterfalls (72.7 %, 75 % and 89.5 %, respectively)(Figure 2.12.a., first four columns). However, juvenile exposure to $MnCl_2$ combined with T_3 and T_4, significantly decreased the percentage of immature fish that overcame the waterfall (33.3 %, Chi-Square test, $p = 0.001$) (Figure 2.12.a., column 5). This decrease could not be observed when the immature fish were injected with GnRH before testing (75 %) (Figure 2.12.a., column 6).

Among immature fish that overcame the waterfall (Figure 2.12.a), the percentage that chose the odorant treated side is shown in Figure 2.12.b. Since the set-up offered two choices a binomial test has to be used to analyze results and 50 % of the fish should enter each compartment, if the odorant does not influence their behavioural response.

Figure 2.12.a

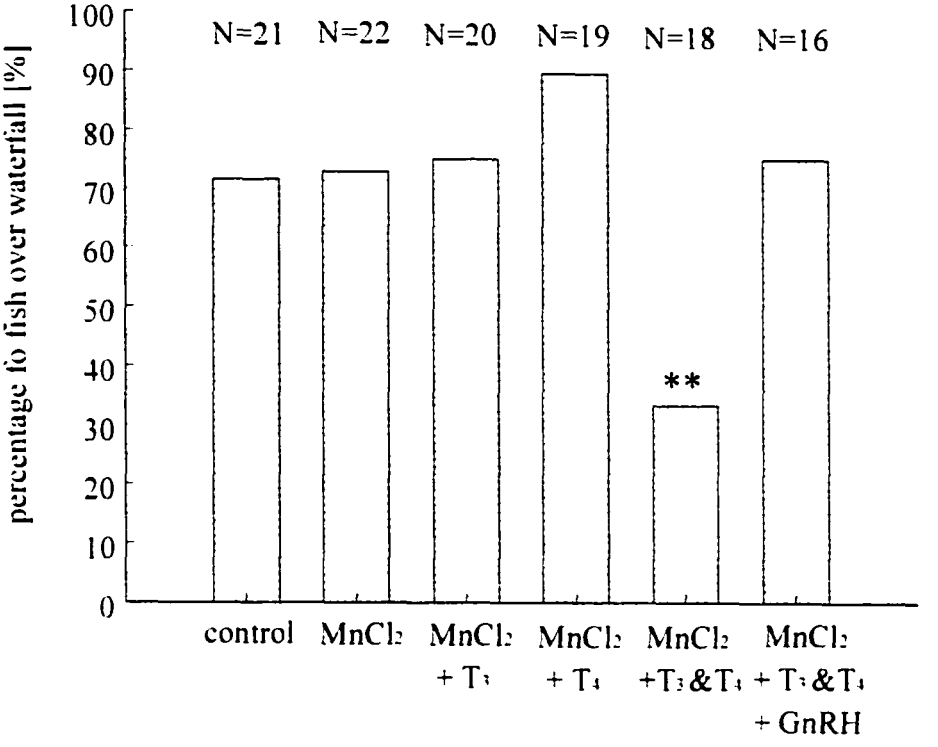


Figure 2.12.b.

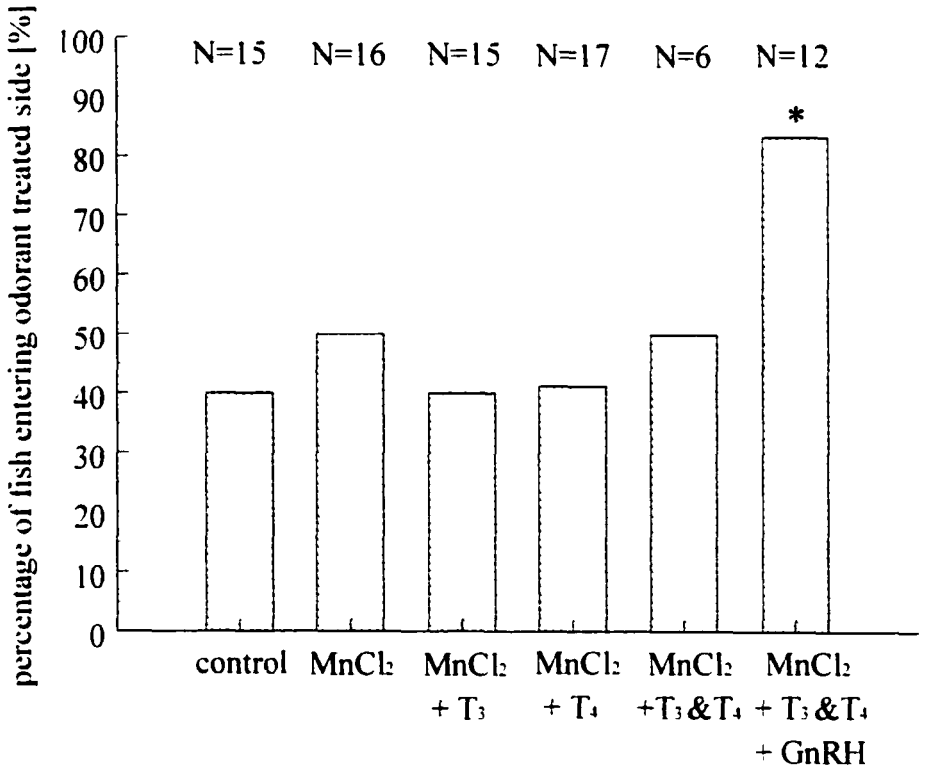


Figure 2.12. a. & b. (legend next page)

Figure 2.12. a. & b: Behavioural response of immature sockeye salmon recorded in the y-maze waterfall test in the presence of $MnCl_2$ in one of the two waterfalls. Figure 2.12.a. shows the percentage of fish that overcame either one of the two waterfalls and Figure 2.12.b. shows how many of those fish chose the odorant treated side. Figure 2.12.a.: Juvenile exposure to $MnCl_2$ (72.7 %, Chi-Square test $p = 0.557$) $MnCl_2$ and T_3 (75 %, Chi-Square test $p = 0.475$) and $MnCl_2$ and T_4 (89.5 %, Chi-Square test $p = 0.061$) did not lead to a significant change in the percentage of immature fish that overcame the waterfall two years later. Juvenile exposure to $MnCl_2$ combined with T_3 and T_4 decreased the percentage of immature fish that overcame the waterfall significantly (33.3.%, Chi-Square test $p = 0.001$). This decrease was reversed when the fish were injected with GnRH before testing (75 %, Chi-Square test $p = 0.503$).
Figure 2.12.b.: Among the immature sockeye that overcame the waterfall the percentage of fish that entered the odorant treated side was not significantly different from 50 % in the control group (40 %), $MnCl_2$ exposed (50 %), $MnCl_2 + T_3$ (40 %), $MnCl_2 + T_4$ (41.1 %) and $MnCl_2 + T_3$ & T_4 (50 %). When injected with GnRH before testing, 83.3 % of the $MnCl_2 + T_3$ & T_4 group chose the odorant treated side. This value was significantly different from the expected 50 % value (Binomial test $p = 0.039$).

The odorant did not change the randomly assumed ratio of 50 % of the fish that enter each of the two waterfall compartments in cohorts with the following juvenile exposure history: no treatment, control fish (40 %); exposure to $MnCl_2$ (50 %); exposure to $MnCl_2$ and T_3 (40 %); exposure to $MnCl_2$ and T_4 (41.1 %); exposure to $MnCl_2$ combined with T_3 and T_4 (50 %) (Figure 2.12.b., column 1-5). However, when the latter group ($MnCl_2$ & T_3T_4 combined) was injected with GnRH before testing, significantly more (83.3 %) sockeye salmon entered the odorant treated side (binomial test, $\alpha = 0.039$). In conclusion, GnRH increased the number of immature, by way of T_3T_4 treatment, $MnCl_2$-imprinted three-year-old sockeye salmon that overcame a waterfall. In the same cohort, GnRH also increased the percentage of fish that entered the $MnCl_2$-scented compartment.

Figure 2.13.a.

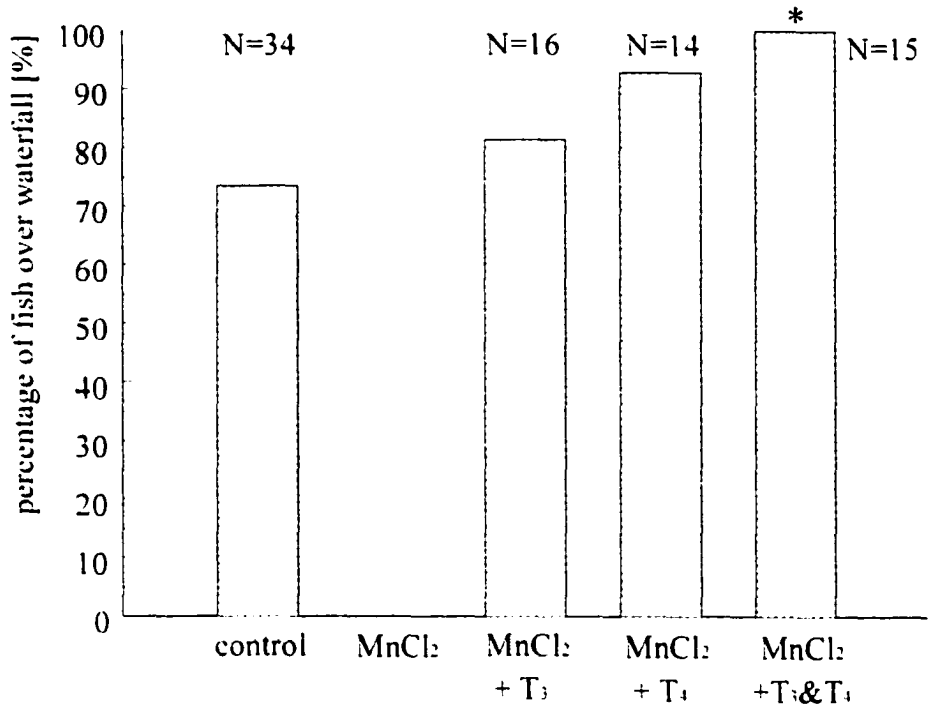


Figure 2.13.b.

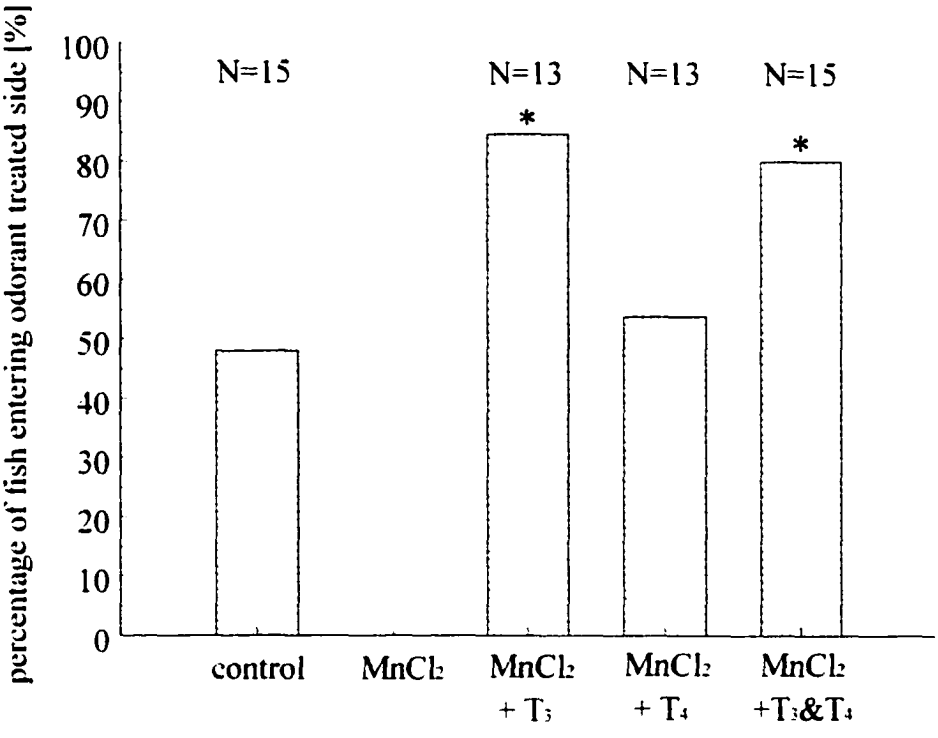


Figure 2.13.a. & b. (legend next page)

Figure 2.13.a. & b: Behavioural response of mature sockeye salmon recorded in the y-maze waterfall test in the presence of $MnCl_2$ in one of the waterfalls. Figure 2.13.a. shows the percentage of fish that overcame either one of the two falls and Figure 2.13.b. shows how many of those fish entered the odorant treated side. All groups with the exception of the control group were exposed to the odorant or odorant and hormone treatment for 30 days, starting 90 days after first morphological signs of smolting. Figure 2.13.a.: The group that experienced juvenile exposure to $MnCl_2$ only, died and was therefore lost for testing. Juvenile exposure to $MnCl_2$ and T_3 (81.3 %) or $MnCl_2$ and T_4 (92.9 %) did not lead to a significant change in the percentage of mature fish that overcame the waterfall. For mature sockeye that were exposed to $MnCl_2$ and a combination of T_3 and T_4 the percentage increased significantly (Chi-Square test, $p = 0.013$) to 100%, when compared with the control group percentage (73.5 %). Figure 2.13.b.: Among mature sockeye salmon 48 % of the control group and 53.8 % of the $MnCl_2$ + T_4 treated group jumped up the odorant treated waterfall. Juvenile treatment with $MnCl_2$ and T_3 (84.6 %) and $MnCl_2$ combined with T_3 and T_4 (80 %) resulted in a significant increase of sockeye that entered the odorant treated side at maturity (binomial test for $MnCl_2$ and T_3 group $p = 0.022$, for $MnCl_2$ and a combined T_3 and T_4 treatment $p = 0.035$).

The behavioural response of three-year-old mature sockeye in the Y-maze waterfall is illustrated in Figure 2.13. Figure 2.13.a. shows the number of fish that overcame the waterfall and Figure 2.13.b. the percentage of jumpers that entered the odorant treated side plotted against four different juvenile odorant and hormone exposure groups.

Column one in Figure 2.13.a. represents the 25 out of 34 or 73.5 % of the mature control fish that overcame the waterfall. Consequently we used this percentage as the expected value for the Chi-Square test. Mature sockeye salmon that were treated with $MnCl_2$ and T_3 or $MnCl_2$ and T_4 overcame the waterfall more often than the control group (81.3 % and 92.9 %, respectively) but the difference was not statistically significant. In contrast, 100 % of the fish that had been challenged with a combination of T_3 and T_4 during the exposure to $MnCl_2$ overcame one of the waterfalls (Figure 2.13.b., fourth

column). This jumping frequency was significantly higher than the one observed for the control group (Chi-Square test $p = 0.013$). Among mature sockeye salmon that overcame one of the waterfalls, 48 % of the control group and 53.8 % of the $MnCl_2$ and T_4 treated fish entered the odorant treated side (Figure 2.13.b.. first and third column). These percentages were not significantly different from the expected value of 50 %. Mature sockeye salmon that had experienced juvenile exposure to $MnCl_2$ and T_3 or $MnCl_2$ and a combination of T_3 and T_4, entered the odorant treated side with a frequency of 84.6 % and 80 % respectively (Figure 2.13.b.. second and fourth column). These frequencies were significantly higher than 50% (binomial test p-values are 0.022 for $MnCl_2$ and T_3. 0.035 for $MnCl_2$ combined with T_3 and T_4 treatment). The presence of $MnCl_2$ hence attracted mature sockeye salmon if they had been exposed to the odorant plus T_3 or a combination of T_3 and T_4, at imprinting.

II.4. Discussion

The main purpose of this study was to determine whether the two factors, developmental stage or hormonal status, could facilitate olfactory imprinting during juvenile exposure to an odorant. My behavioural experiments did not provide any evidence that odorant exposure by itself, at any of the tested juvenile developmental stages was sufficient to initiate imprinting under hatchery rearing conditions. In contrast, the behavioural evidence gathered, indicates that treatment with a combination of the two thyroid hormone forms (T_3 and T_4) or T_3 only can initiate the imprinting process to an odorant.

To avoid the criticism that Hasler and Scholz (1983) received for their choice of imprinting odorant (morpholine), which was shown to be a non-specific attractant for salmonids (Mazeaud, 1981/1982), I first determined the general behavioural response to candidate odorants for use in the imprinting experiments. When fish are naturally attracted to or repelled by an odorant, they will react to the odorant independent from previous exposure history and behavioural results are difficult to interpret. Sockeye salmon in my experiments were naturally repelled by $CuCl_2$ and cholic acid and did not react behaviourally to $MnCl_2$, L-glutamine and $BaCl_2$. In the first behavioural experiments, it became also apparent that using two behavioural arenas that differed markedly in the behavioural responses that fish displayed during testing, proved to be beneficial. The low energetic investment necessary to respond to an odorant in the behavioural trough, made it easy to detect whether an odorant was an attractant or a repellent. In the commonly used Y-maze set-up (Hasler & Scholz, 1983; Dittman et al., 1994; Dittman et al., 1996) those subtle changes in behaviour could not be detected. This

is not to say that Y-maze set-ups cannot serve to demonstrate behavioural reactions to odorants when fish are highly motivated to migrate at final maturation. Once the choice of odorants was narrowed down to the more suitable representatives of certain odorant groups and the functionality of the behavioural arenas was established, I proceeded to test for the effect of juvenile odorant exposure. Juvenile odorant exposure on its own could not trigger imprinting although exposure periods ranged from one month to the whole juvenile development past the PST stage and thus covered all stages that have been suggested for natural imprinting (Hasler & Scholz, 1983; Dittman et al., 1996; Nevitt & Dittman, 1998).

But why do sockeye salmon in nature imprint to an odorant that they have been exposed to? To answer this question, it was important to emphasize that the increase in T_3 and T_4 plasma levels found in salmon in nature, are largely suppressed in captivity (Youngson & Simpson, 1984, this study). I therefore tested whether exogenous challenges with one or both types of thyroid hormones would allow fish to imprint. Separate treatment with T_4 proved to be inadequate but a combined treatment with T_4 and T_3 led to olfactory imprinting at two developmental stages that are not naturally related to the imprinting process. Thus, I could initiate imprinting independent from the commonly accepted developmental stage of PST and suggest that the often mentioned T_4 on its own is not able to provide the physiological background conditions for imprinting (Hasler & Scholz, 1983; Dittman et al., 1994; Dittman et al., 1996). Moreover, imprinting was achieved in a stimulus-poor hatchery environment, which excludes the possibility that changes in current, temperature, food composition or visual environment, all suggested as possible imprinting triggers, might have stimulated the process in this study.

Through which course of action the combined T_4T_3 treatment triggered the olfactory imprinting process is unknown. Since thyroid hormones act on almost every organ in an organism at one point (see introduction of Chapter III), the hormones might act directly at the level of the olfactory epithelium or more centrally along the olfactory neuronal pathways. The possible imprinting related sensitization of olfactory receptor neurons has been suggested by Nevitt et al. (1994) and Dittman et al (1997). While the experimental evidence gathered by these authors is conclusive, it is difficult to explain how this peripheral sensitization is maintained when olfactory receptor neurons are turned over and replaced by new receptor cells (Farbman, 1994). It is also hard to explain how Nevitt et al. (1994) were able to pick the specific receptor cells that could bind their imprinting odorant for single cell recordings out of millions of olfactory receptor cells that should have not been able to bind the imprinting odorant (Buck & Axel, 1991). I believe more studies investigating the turn-over rate of olfactory receptor neurons and the specificity of each receptor cell to one odorant in imprinting fish are necessary.

In addition to the preceding results from the behavioural trough, I gathered the following behavioural evidence for T_3T_4 based imprinting in the Y-maze waterfall. First my experimental efforts were focused on the determinants of the motivation to overcome a waterfall. Waterflow significantly influenced jumping performance and was set optimally to 250 L/min. Then possible effects of gender and state of maturity were determined. While gender did not seem to influence jumping performance, mature fish were significantly more motivated to jump up a waterfall than immature fish. The next experiment was aimed at singling out the agent, co-occurring with maturation, that could initiate the motivational increase to overcome a waterfall. Sato et al. (1997), found GnRH

treatment, possibly by way of heightened steroid hormone levels, to be effective in motivating sockeye salmon to migrate faster to their natal stream. Therefore, it appeared reasonable to test whether GnRH treatment might also increase the motivation to overcome a waterfall in this study. When testing for jumping motivation, I also recorded the extent of the motionless period of acclimatization to a new environment, typically occurring after transfer into a new tank. During this period, fish display fast and strong opercular movement, a typical sign of stress. Hence, I expected this period to increase with increasing stress to the fish, in the second of two tests. For control fish this assumption was verified but GnRH effects possibly overrode this stress response since GnRH injected fish showed no increase in their time of acclimatization between test one and test two. GnRH might counteract the rise of adrenaline levels experienced at times of high stress (Joy et al., 1998).

The number of passes through, and jumps up the waterfall increased significantly in the second test subsequent to GnRH injections but stayed unchanged in the saline injected control group. GnRH seemed to increase either the motivation to overcome a waterfall, motor neuron activity or a combination of both factors. It is highly speculative to discuss the nature of interaction between GnRH and the central nervous system that leads to the observed behavioural changes. GnRH might interact with centers in the CNS that are involved in the detection or processing of olfactory information or it might act indirectly through a multimodal sensory center (Wulliman, 1998). Alternatively, GnRH may interact with a hormone-sensitive center in the telencepahlon in combination with a feedback system that regulates the function of the olfactory bulb through centrifugal fibers, as proposed by Hara (1967b). The GnRH neuron network seems to fulfill all of the

above mentioned requirements but electrophysiological recordings have not yet identified stimulation of the olfactory system by GnRH neurons (Hara, personal communication). Evidence for direct neuromodulatory action of GnRH in the olfactory epithelium has just recently been reported by Okubo et al. (2000) who discovered GnRH binding sites on ORNs and by Eisthen et al. (2000) who observed GnRH triggered sensitivity modulation in ORNs.

In the last experiment, my aim was to relate juvenile odorant and hormone exposure histories to behavioural reactions of three-year-old immature and mature sockeye salmon in the motivationally demanding Y-maze waterfall. Firstly, the number of fish in each group that would overcome either one of the waterfalls was recorded to monitor differences in motivation to jump and secondly, the number of fish that chose the odorant treated site was monitored. Immature sockeye previously exposed to $MnCl_2$ or to $MnCl_2$ and T_3 or T_4 did not differ significantly from the control group in the percentage of fish that overcame the waterfall and the percentage of fish that chose the odorant treated waterfall did not differ significantly from the expected 50 %. Nevertheless, a significant lower number of immature sockeye that experienced a combined T_3 and T_4 treatment at the artificial imprinting stage surmounted either one of the waterfalls when their imprinting odorant was present. The same odorant and hormone exposure record that had led to a behavioural response, that was interpreted as odorant recognition in the behavioural trough, led to an avoidance of waterfalls when confronted with the imprinting odorant. In nature, some of the migratory salmonid species are confronted with the odorant of their natal stream from time to time. Nevertheless, they do not enter their stream of origin before they reach the stage of maturity. It has been hypothesized

that speciation in sockeye salmon can be facilitated by temporal reproductive isolation within the same spawning stream (Tallman & Healey, 1994; Wood & Foote, 1996). If it is desirable to minimize gene flow between different species or even morphs of the same species than precise timing of the spawning migration is crucial. Avoidance of the natal stream at times outside the stock-specific spawning season therefore appears reasonable. Moreover, premature migration into mostly food scarce and energy demanding natal streams does not seem to be advantageous for the growth of fish. If it is assumed that the behavioural avoidance reaction of immature sockeye salmon in this study was intentional, then mature and imprinted fish should be motivated to jump up the waterfall and choose the odorant treated side. When I first tried to mimic maturity by a GnRH injection this hypothesis was verified: The percentage of fish that overcame the waterfall increased significantly and a significant majority of these fish chose the odorant treated side. Therefore GnRH not only increased the motivation to overcome a waterfall but it also enhanced olfactory recognition of the odorant that the fish had been imprinted to. Furthermore, it was reconfirmed that a combination of T_3 and T_4, parallel to the juvenile odorant exposure, could facilitate olfactory imprinting. The effects of a combined T_3 and T_4 treatment may also have caused an additional increase of T_3 when compared with the group that was treated with T_3 only. This alternative hypothesis is nevertheless dependent on the conversion of T_4 to T_3, which is assumed to be suppressed by exogenous T_4 treatments (Eales, 1995). The fact that T_3 treatment during juvenile odorant exposure led to behavioural patterns in the Y-maze waterfall of three-year-old sockeye that were interpreted as odorant recognition based on olfactory imprinting, supports this hypothesis.

In conclusion, the evidence accumulated in my behavioural tests led to the suggestion that combined T_3 and T_4 treatment facilitates juvenile olfactory imprinting. Either both forms of thyroid hormones or in a less reliable fashion T_3 treatment initiated imprinting when fish were tested in one of the two behavioural set-ups. I therefore recommend combined T_3 and T_4 treatments for future imprinting experiments. My data furthermore suggest that T_4 is not a convincing imprinting facilitator and that the hormonal state of maturity is crucial for the recognition of an imprinting odorant.

Chapter III: Thyroid and gonadotropin hormones in the context of olfactory imprinting [3].

III.1. Introduction: Thyroid hormones and Gonadotropins during salmonid ontogeny

Thyroid and gonadotropin hormones affected the outcome of my behavioural experiments in a significant way. They independently or interactively facilitated juvenile olfactory imprinting that lead to recognition of the imprinting odorant in adult fish. Therefore, I decided to investigate the hormonal background and the possible interaction of thyroid and GnRH systems (Figure 3.1) in our experimental fish.

Background: Thyroid hormones

In its normal state, the thyroid system is controlled by the pituitary, which in turn is under neurosecretory control of the hypothalamus (Cyr & Eales, 1996). It is still unclear, which chemical factors are released by the hypothalamus to establish the brain-pituitary axis but it is clear that the pituitary controls the thyroid gland via thyroid-stimulating hormone (TSH) (Eales, 1995). The thyroid, driven by TSH, synthesizes and releases mainly L-thyroxine (T_4) (Figure 3.2.b.) which is partly converted to 3,5,3`-triiodo-L-thyronine (T_3) (Figure 3.2.b.). T_3 has about a ten times higher affinity to nuclear

[3] This study was carried out in collaboration with Bruce Adams, University of Victoria and Dr. Geoff Eales, University of Manitoba.

receptors of target tissue cells than T_4 (Cyr & Eales, 1996; Eales, 1995) and T_4 to T_3 conversion is known to occur in many different tissues of teleost fish (Eales & Brown, 1993). Among these tissues the liver seems to be the most important T_3 donor to blood plasma (Morin et al., 1993). Sensory target tissues like the olfactory epithelium and the retina have also been suggested as locations of deiodinase activity (Morin et al., 1995; Cyr & Eales 1996) but supporting data has only recently been provided for the retina (Orozco et al., 2000). In general, a variety of essential processes are affected by thyroid hormones in vertebrates (Cyr & Eales, 1996). Examples are: growth (Hazon & Balment, 1998), control of metamorphosis (Denver, 1998), alteration of visual pigments (Browman & Hawryshyn, 1992; Alexander et al., 1994), osmoregulation (Hoar, 1988), increase in dendritic arborization of neurons (Rami et al., 1986 a & b; Arnold, 1992) and the modulation of olfactory sensitivity (Morin et al., 1995, 1997). The common thread through these examples is the mode of action. After release from binding proteins in the bloodstream, T_4 enters the target cell by diffusion through the cell membrane (thyroid hormones are lipophilic) and by facilitated diffusion involving membrane carriers (Eales, 1995). Within the cell, most of the T_4 is converted to T_3 by enzymatic activity of T_4 outer-ring deiodinase (Figure 3.2.). The resulting T_3 binds to nuclear receptor sites that control the transcription of genes affecting cell differentiation (Eales & Brown, 1993; Eales 1995). In response to high T_4 levels in target tissues, T_4 inner-ring deiodination can act as an autoregulator and produce inactive reverse T_3 (rT_3) (Figure 3.2.). This is believed to be a disposal pathway at times of T_4 saturation (Eales & Brown, 1993). To date, nuclear T_4 receptors have not been identified (Eales, 1995) and it is well known that T_4 challenges can initiate the thyroid system to switch to the disposal mode mentioned in

the last sentence. This can lead to a drastic decrease of T_3 available to target tissues (Morin et al., 1995). Therefore it is crucial to monitor T_4 and T_3 when attempting to modify the thyroid system.

While approaching maturation, the stage when odorant recognition is important for homing, T_4 plasma levels increase briefly, then gradually decrease to a low level (Biddiscombe, 1983). Oestradiol can act as an inhibitor for the thyroid gland (Figure 3.1) and consequently an increase in oestradiol, experienced at maturation, leads to a decrease in T_4 synthesis and release. Low T_4 blood plasma levels do not necessarily mean low T_3 availability for target tissues, if increased deiodinase activity compensates for reduced T_4 availability. For T_3-driven gene expression in the olfactory epithelium to increase olfactory sensitization at maturation, there must be a link that connects the onset of maturation with increased thyroid availability in target tissues. In this study, it was therefore investigated whether GnRH might link the beginning of migratory behaviour with increased deiodinase activity in sensory or non-sensory tissues.

Background: GnRH

Gonadotropin injections have been shown to enhance the occurrence of behavioural patterns that are related to homing in salmon (Hasler & Scholz, 1983, Sato et al., 1997, this study). The gonadotropin system should therefore be considered when searching for agents that could enhance migratory motivation and/or sensitization of the olfactory system towards imprinting odorants. Gonadotropins primarily regulate reproduction and the output of sex steroids. GnRH production early in ontogeny also coincides with the onset of GnRH neuron expression, which starts in the olfactory

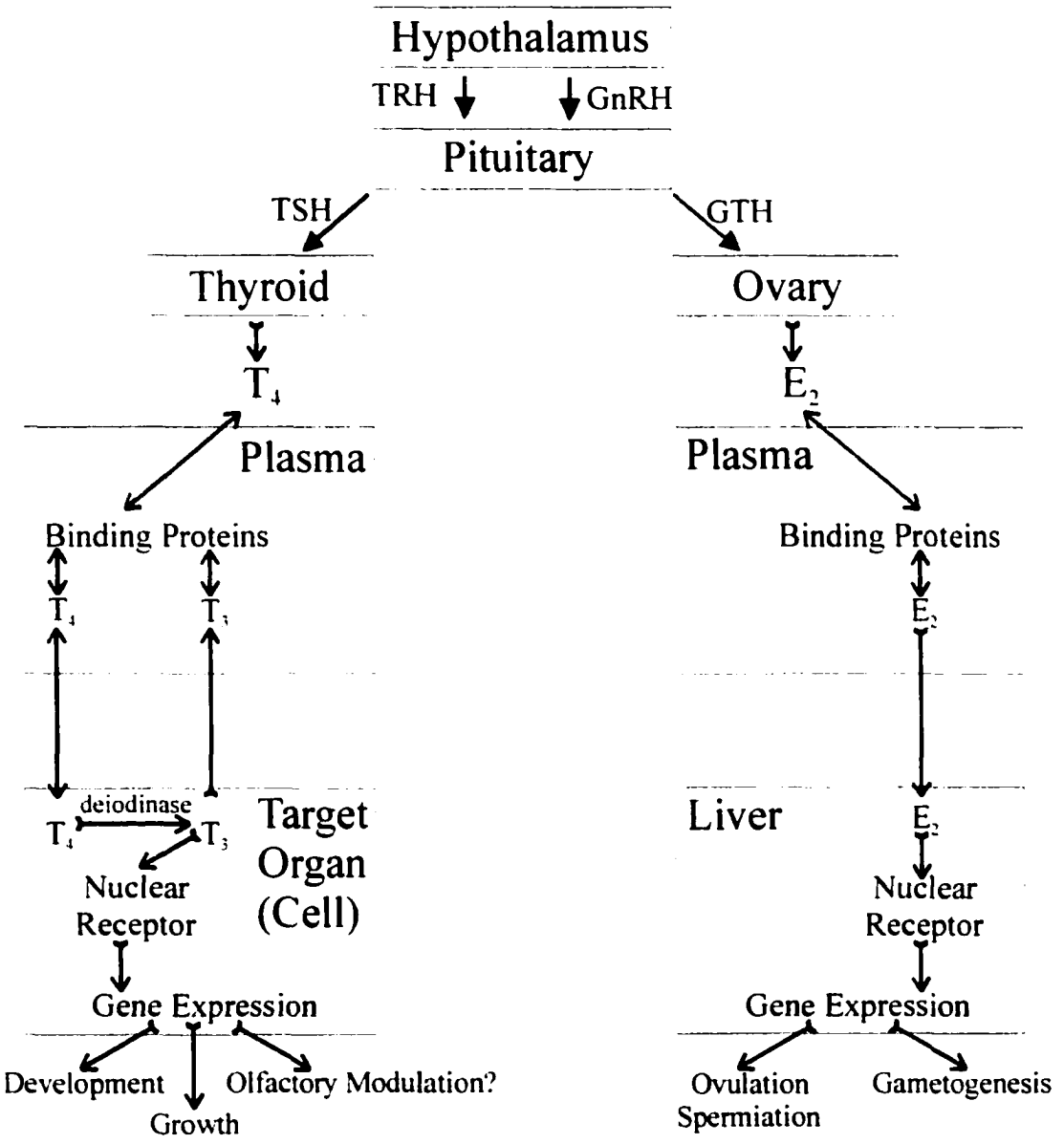


Figure 3.1 : The thyroid and the reproductive endocrine systems. Interaction between Oestradiol (E_2) and the thyroid system and T_3 and the reproductive system are know but not understood in detail (after Eales & Brown, 1993; Cyr & Eales, 1996).

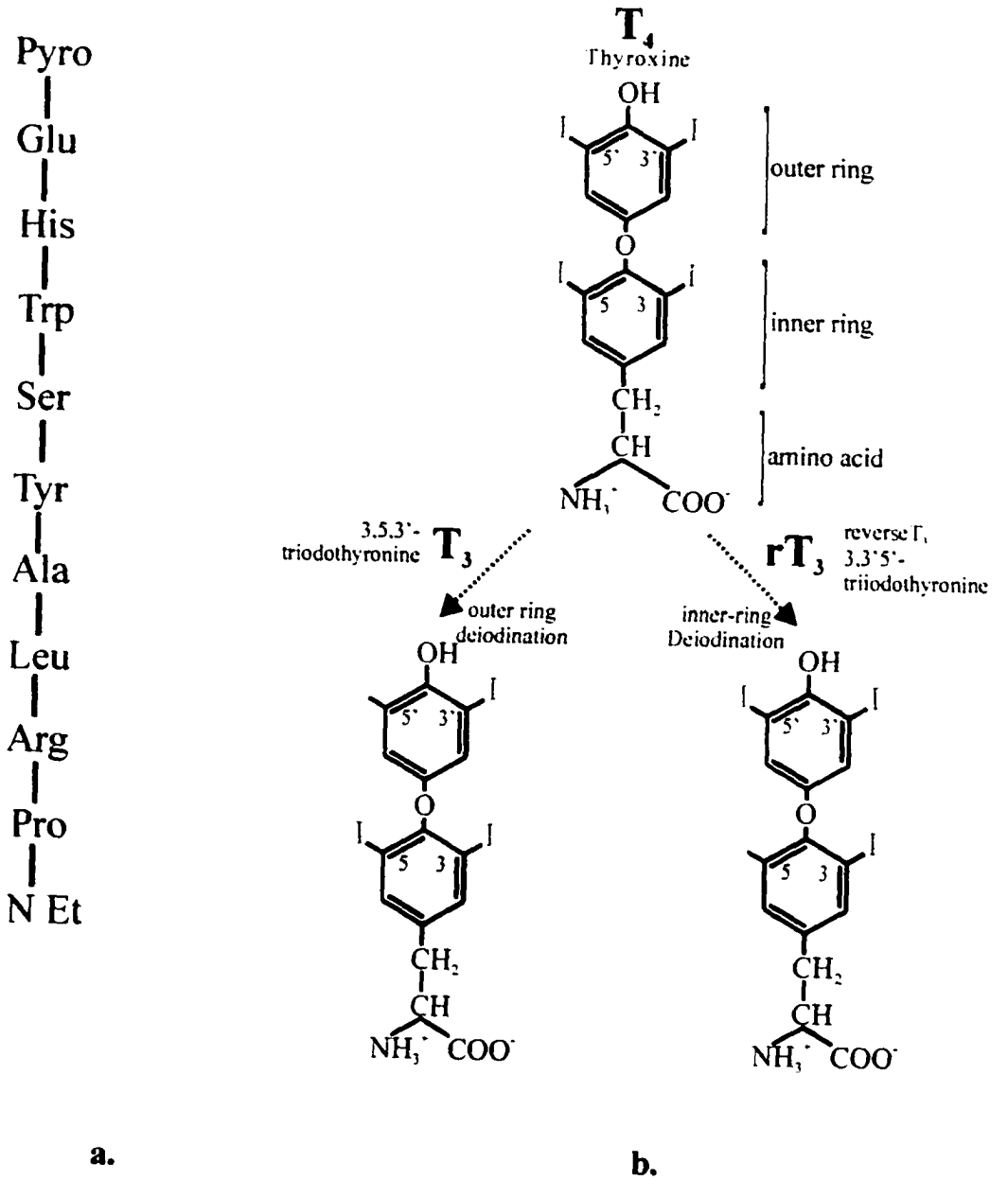


Figure 3.2: Molecular structure of hormones: a. structure of the D-Ala-6-N-Ethyl mammalian gonadotropin-releasing hormone analogue used in this study, b. structure of thyroxine (T_4), 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3, rT_3)

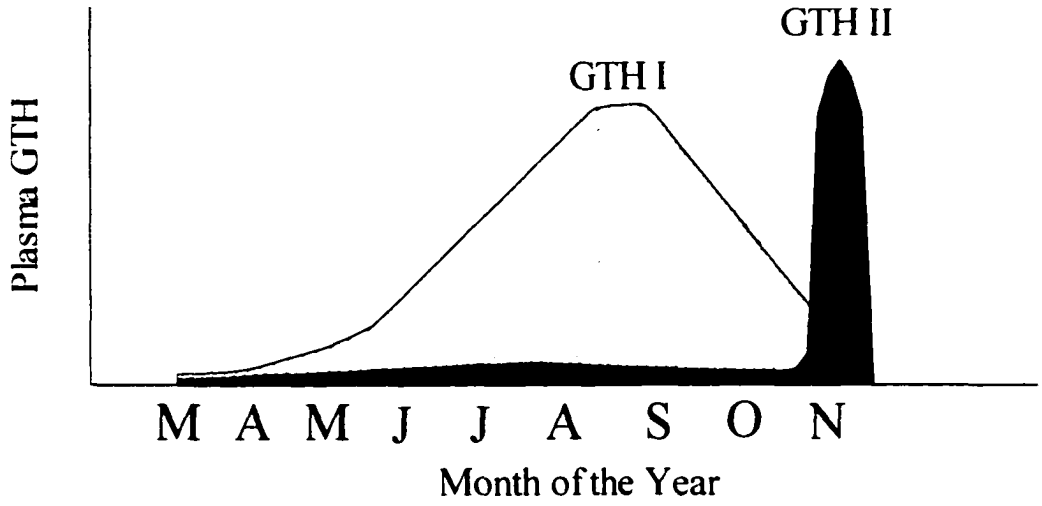


Figure 3.3: Changes in plasma level of GTH I and GTH II in coho salmon during the months prior to maturation (after Dickhoff & Swanson, 1990).

placode of sockeye salmon only 19 days after fertilization (Parhar et al., 1995). GnRH neurons might therefore affect the peripheral olfactory system at the earliest stages of development, perhaps initiating or maintaining imprinting processes. The gonadotropin system is controlled by fibers originating from hypothalamic centers that innervate gonadotrope cells in the pituitary gland and thus regulate the release of gonadotropin hormone (GTH) via GnRH secretion. Dopamine acts as a direct and indirect (via the hypothalamus) inhibitor of GTH release from the pituitary in goldfish and rainbow trout and might therefore also be responsible for GTH regulation within the brain of other fish species (Chang et al., 1990, Vacher et al., 2000). GTH in fish can be found in two forms,

GTH I and GTH II (Suzuki et al. 1988c). GTH I promotes vitellogenesis and spermatogenesis long before spawning in coho salmon (Figure 3.3) (Dickhoff & Swanson, 1990). Shortly before final maturation, the GTH I plasma level declines as the GTH II level rises, initiating ovulation and spermiation (Dickhoff & Swanson, 1990). The most drastic increase of the GTH I level hence coincides with the onset of homing behaviour in coho salmon (Dickhoff & Swanson, 1990) and it seems likely that GTH I levels are elevated in other anadromous salmon species at the same stage. GnRH increased the motivation of sockeye salmon to migrate to their natal stream (Sato et al., 1997) and recently it has been reported that GnRH receptors are expressed in the olfactory epithelium of the Japanese eel (*Anguilla japonicus*) (Okubo et al., 2000). Together with Eisthen et al. (2000) findings that demonstrate neuromodulatory capabilities of GnRH at the level of olfactory receptor neurons (or ORNs), GnRH appears as a likely candidate to be also involved in olfactory guided homing.

Objectives and experimental approach

My first objective was the monitoring of the status of both forms of thyroid hormones in juvenile sockeye salmon before and in response to exogenous T_3 and T_4 treatment. I thus tried to evaluate how juvenile sockeye salmon respond to artificial T_3 and T_4 challenges before PST. I then waited until the PST stage was terminated and challenged again with both forms of thyroid hormone. The second challenge was carried out at a time when levels of thyroid hormones are naturally low and thus the effect of the challenge could be interpreted without the disturbance of natural hormonal fluctuations.

In the second experiment, I compared thyroid hormone levels of adult immature and mature sockeye salmon between wild fish and fish that were raised in a hatchery environment. The objective was the investigation of possible effects of the stimuli-poor hatchery environment on the thyroid hormone household.

In the third experiment, non-maturing three-year-old sockeye salmon were injected with GnRH, and monitored for corresponding changes in the activity of deiodinase enzymes. This was an attempt to clarify whether thyroxine can be activated or deactivated independently from prevailing blood plasma levels by GnRH in the olfactory epithelium, the retina, the liver and the brain of sockeye salmon.

III. 2. Materials and methods:

For general materials and methods like egg sampling, incubation, rearing, thyroid hormone feed treatment and GnRH treatment see materials and methods of Chapter II.

Experiment 1 and 2:

Blood sampling for thyroid hormone assays

In preparation for blood sampling, sockeye were anaesthetized with tricaine methanesulfonate (MS 222). Fish were then killed by a sharp blow to the head and their tail was amputated for blood collection. The blood was subsequently centrifuged and plasma was drawn off and stored frozen (-60°C) until assayed for T_3 and T_4. To obtain enough plasma for one sample (50µl for T_3 and 25µl for T_4) in the youngest group (six weeks after first feeding, average weight 2.95 g ± 0.55g) two to four fish had to be pooled. When in short supply of plasma, T_3 was measured first, as it is considered to be the physiologically active form of thyroid hormone. In the post-PST and the age 3 groups, individual fish had enough plasma for one assay per fish (average weight post-PST group: 9.61g ± 1.65g; average weight age 2 group: 210g ± 32g).

Radioimmunoassays for free T_3 and T_4

Plasma T_3 and T_4 concentrations were determined by using the radioimmunoassay kits Coat-A-Count® TOTAL T_3 and T_4 (Diagnostic Products Corporation, Los Angeles, California, USA). Both assays are based on competitive protein binding to T_3 or T_4

specific binding sites. The process was started by filling duplicate 50 µl plasma samples for T_3 and 25 µl for T_4 into corresponding specific antibody-coated tubes for incubation with a ^{125}I-labeled T_3 or T_4 tracer. After incubation, decantation and drying, radioactivity of the tubes was measured in a gamma counter and the amount of T_3 or T_4 in the plasma sample was determined by interpolation from a standard curve. Low radioactivity counts implied high thyroid hormone concentrations in the plasma since the radioactively labeled T_3 or T_4 and the plasma T_3 or T_4 were competing for the specific binding sites.

Sample Timing

Sample and hormone treatment timing for juvenile sockeye salmon:

T_3 levels of juvenile sockeye salmon were determined five weeks after first feeding (day 175 after fertilization), six weeks after first feeding (day 182 after fertilization), 83 days after the onset of PST (day 343 after fertilization) and 90 days after the onset of PST (day 350 after fertilization). The samples six weeks after first feeding and 90 days after the onset of the PST were taken three days after indivudual cohorts of fish had been challenged with T_3 , T_4 or a combined T_3T_4 challenge.

Serum T_4 levels were sampled 90 days after the onset of PST (day 350 after fertilization) and 97 days after PST (day 357 after fertilization). Three days before the first T_4-sampling date three groups of fish had been treated with T_3 , T_4 or T_3T_4 combined.

Sample and hormone treatment timing for adult sockeye salmon:

Blood samples from wild sockeye salmon were taken on September 22nd, 1996 from two locations at Fulton River Spawning Channel, Babine Lake, northern British

Columbia, Canada. One group of fish was sampled at the mouth of Fulton River when approaching maturity. At the time of blood sampling gonadal products were still firm indicating that fish had not reached final maturity. The other set of blood samples was taken from fish on the spawning beds within the channel during spawning. Eggs and sperm in this group were readily released and some fish had started to spawn. Hatchery reared sockeye were either sampled at age three (day 1092 after fertilization) without any signs of maturity or at the same age fully mature (eggs or sperm were released upon handling).

Experiment 3:

Deiodinase assay tissue sampling

Tests for deiodinase activity were carried out on three-year-old immature sockeye salmon. Tissue samples were dissected shortly after euthanasia and stored in liquid nitrogen to halt deterioration of enzymatic processes. Brain, liver, retina and olfactory epithelium were sampled for the deiodinase assay. Liver represents the tissue with the highest deiodinase activity in fish and served as a positive control. Parallel to the deiodinase activity, T_4 and T_3 blood plasma levels were determined in the GnRH challenged and control fish. Following Eales et al. (1999), corresponding tissues from different fish within the same treatment groups were pooled when less than 0.5g of the respective tissue could be collected from an individual fish.

Deiodinase activity assay

General principle: T_4 is converted to T_3 by removal of one of the two outer ring iodine molecules (I^-). When the iodine is detached from the 5' position within the outer

phenyl ring by the enzymatic action of the deiodinase, equal molar quantities of T_3 and I^- are created (Figure 3.2.). The degree of deiodinase activity can be determined by using [^{125}I] labeled T_4 ($^*T_4$) as a substrate and measurement of [^{125}I] I^- ($^*I^-$) or [^{125}I] T_3 ($^*T_3$) the products of outer-ring deiodinase activity. The deiodinase-rich microsomal fraction within the chosen tissues is used for the assay and deiodinase activity is related to the amount of microsomal protein. The resulting deiodinase activity is therefore expressed in fmol T_4 deiodinated • h^{-1} • mg microsomal $protein^{-1}$.

Statistical analysis for experiment 1, 2 and 3

Sample data for each treatment group were examined for consistency with the normal distribution (Shapiro-Wilk test) and compared for equality of variance (Levene test) before testing for differences in mean concentrations with the analysis of variance (ANOVA) or an independent sample t-test.

III. 3. Results:

Calibration curves:

The T_4 calibration curve (Figure 3.4. a.) covered values of radioactive counts that were well within the range of the values of results obtained from my own sockeye blood plasma samples (7,000 to 19,000 counts). The extra effort to increase resolution at the lower count-level was necessary to accommodate the human RIA kits to the, in comparison to human T_4 blood plasma levels, lower T_4 levels of fish. The range of radioactivity counts in response to the T_3 standards (Figure 3.4.b.) that came with the RIA kit were fitting within the range of counts that were found in blood plasma levels (3000 to 8000 counts). Thus, T_3 standards were taken as is and did not need extra dilution steps to increase resolution.

T_3 levels in juvenile sockeye salmon

No significant change within the T_3 blood serum levels of hatchery-reared control fish was detected over the sampling period and measured concentrations remained between 2.5 and 3 ng • ml^{-1} (Figure 3.5.a.). T_3 blood serum levels did not change significantly in response to a T_4 challenge (Figure 3.5.a). However, T_3 levels increased significantly to 6.41 ± ng • ml^{-1} following a T_3 challenge six weeks after first feeding and to 7.49 ± 0.82 ng • ml^{-1} following a T_3 challenge 90 days after the onset of PST. T_3 levels also rose significantly in response to a combined T_3T_4 challenge (12 ppm T_3, 120 ppm T_4) six weeks after first feeding (9.47 ± 0.83 ng • ml^{1}) and three month after PST (7.95 ± 0.63 ng • ml^{-1}).

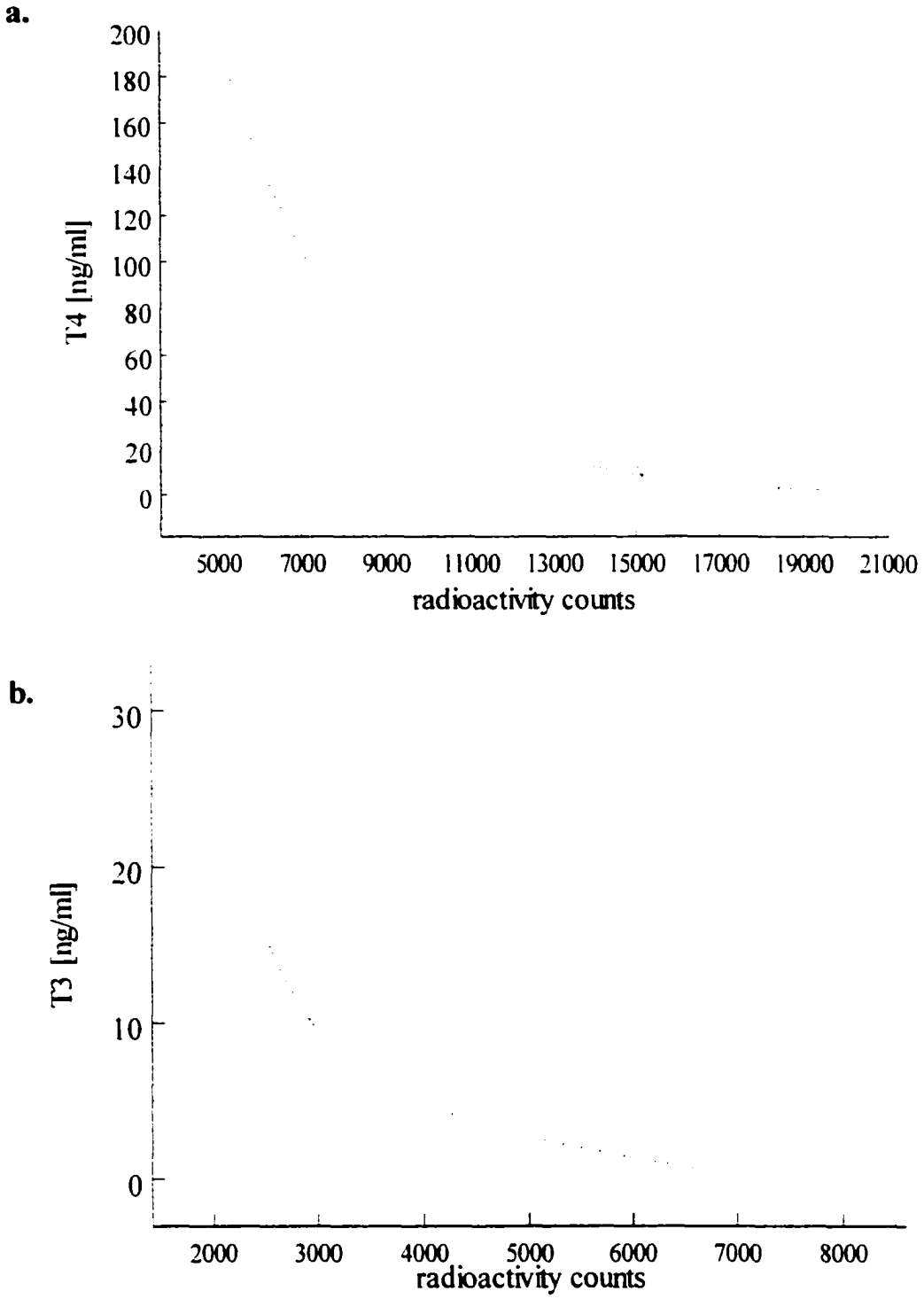


Figure 3.4.: a. Calibration curve for T_4 standards. b. Calibration curve for T_3 standards

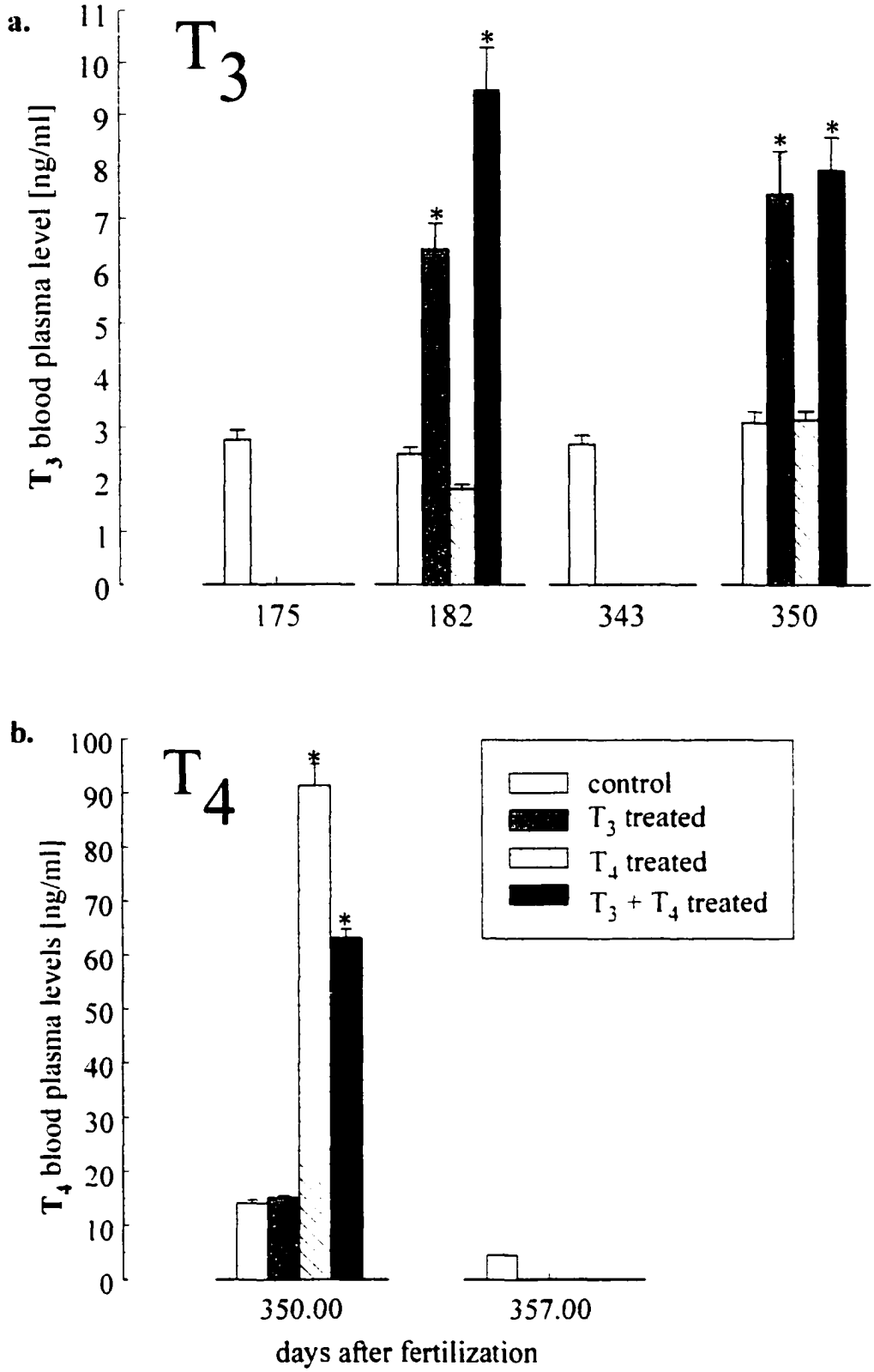


Figure 3.5.a & b (legend following page)

Figure 3.5.a & b: Figure 3.5. shows T_3 (a.) and T_4 (b.) blood plasma levels in juvenile sockeye salmon. Bar colours and patterns refer to: untreated controls (open bar), T_3 treated (gray), T_4 treated (black stripes) or treated with T_3-T_4 combined (black). The legend applies to both graphs. Times (x-axis) of blood sampling are shown in days after fertilization. Figure 3.5.a: The sample at day 175 was taken one day before any hormonal treatment and five weeks after first feeding. The sample at day 182 was taken 6 days after the start of the 30 day treatment with thyroid hormones (T_3, T_4 or T_3T_4 combined) and six weeks after first feeding. Day 343 after fertilization was positioned 83 days after the onset of PST and one day before the start of a 30 day treatment with thyroid hormones. Day 350 after fertilization was positioned 90 days after the onset of PST and six days into treatment with the different thyroid hormones. T_3 and combined T_3T_4 treatment increased the T_3 plasma level at both stages significantly, T_4 treatment had no significant effect on the T_3 serum level. Figure 3.5.b: T_4 serum levels were significantly increased by T_4 and combined T_3T_4 treatment. T_3 treatment did not change the T_4 serum levels significantly. Seven days after the hormonal treatment (day 357) the T_4 plasma levels in control fish had slightly decreased. For times of sampling see explanations of Figure 3.5.a. Data points represent the arithmetic mean ± standard error (error bars). Significant differences of treatment means to control means are marked with an asterisk (ANOVA, $p \leq 0.05$)

T_4 levels in juvenile sockeye salmon

T_4 serum levels of control fish (Figure 3.5.b) dropped significantly from 90 days after PST (14.05 ± 0.55 ng • ml^{-1}) to one week later (4.4 ± 0.15 ng • ml^{-1}). It is therefore assumed that the fish were sampled during a time of natural T_4 decline as experienced after the PST (Hasler & Scholz, 1983). T_4 levels (15 ± 0.36 ng • ml^{-1}) were not significantly altered in comparison to controls when the fish were challenged with T_3 (Figure 2.5.b). Both, T_4 and the combined T_3-T_4 challenges elevated T_4 levels significantly to 91.3 ± 3.97 ng • ml^{-1} and 63.1 ± 1.72 ng • ml^{-1}, respectively.

Figure 3.6.a: T_3 and T_4 levels in wild fish

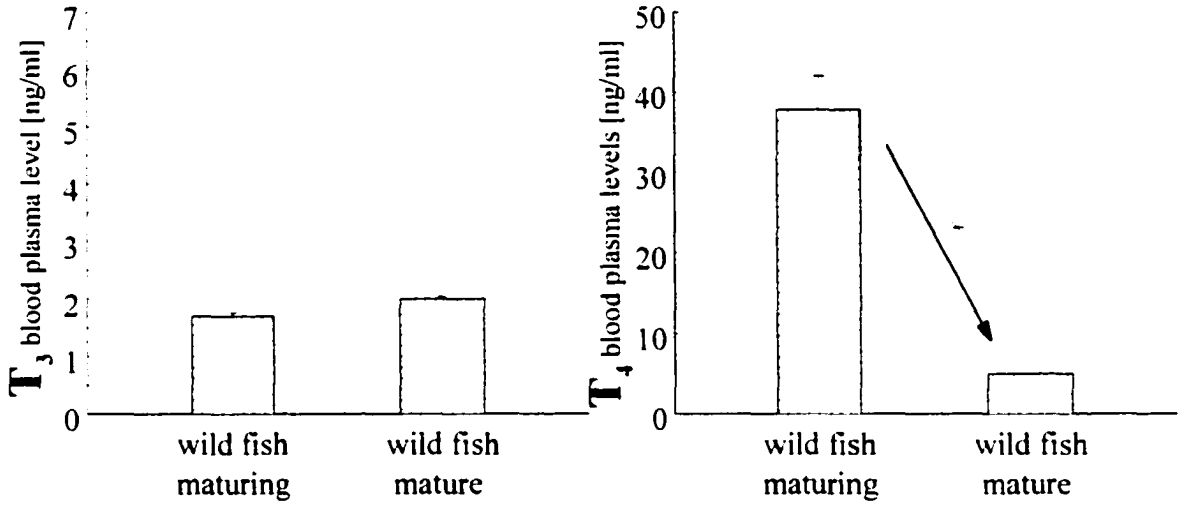


Figure 3.6.b: T_3 and T_4 levels in hatchery reared fish

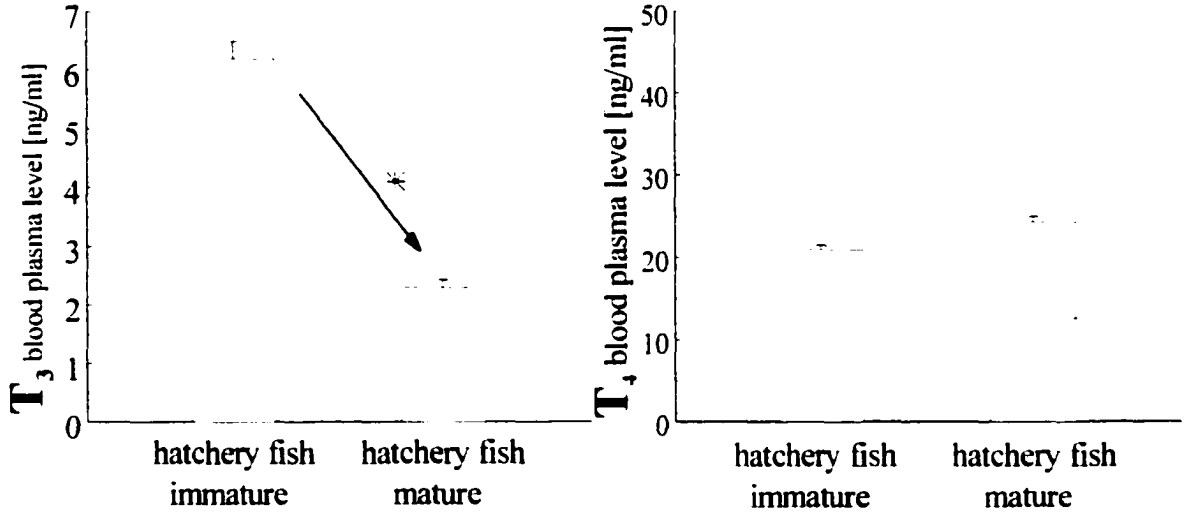


Figure 3.6. (legend following page)

Figure 3.6.a & b: Figure 3.6.a shows T_3 and T_4 blood plasma levels in wild fish and Figure 3.6.b shows T_3 and T_4 blood plasma levels in hatchery-reared sockeye salmon. Asterisks stand for significant (ANOVA, $p \leq 0.05$) differences in the mean concentration between stages. When approaching maturity T_3 blood plasma levels in wild fish did not change significantly (from 1.7 ± 0.06 ng • ml^{-1} to 2 ± 0.04 ng • ml^{-1}) (Figure 3.6.a, left graph) whereas T_4 blood plasma levels dropped significantly (from 37.9 ± 4.2 ng • ml^{-1} to 5 ± 0.08 ng • ml^{-1}) (Figure 3.6.a, right graph)

The T_3 serum level in immature sockeye was significantly higher (6.2 ± 0.29) than the T_3 serum level in mature fish (2.3 ± 0.13 ng • ml^{-1}) (Figure 3.6.b, left graph) whereas the T_4 level was not significantly changed (21 ± 0.46 ng • ml^{-1} in immature fish to 24.3 ± 0.7 ng • ml^{-1} in mature fish) (Figure 3.6.b, right graph).

T_3 and T_4 levels in adult and maturing wild and hatchery reared sockeye

Since immature wild sockeye salmon were not available to me, I chose to sample blood from fish that were approaching maturity to compare to the immature hatchery-reared sockeye salmon.

When approaching maturity, T_3 blood plasma levels in wild fish (Figure 3.6.a) did not change significantly (from 1.7 ± 0.06 ng • ml^{-1} to 2 ± 0.04 ng • ml^{-1}) while T_4 blood plasma levels dropped significantly (from 37.9 ± 4.2 ng • ml^{-1} to 5 ± 0.08 ng • ml^{-1}).

In the hatchery-rearing environment T_3 serum levels in immature sockeye were significantly higher (6.2 ± 0.29) than the T_3 serum levels in mature fish (2.3 ± 0.13 ng • ml^{-1}) whereas the T_4 level was not significantly changed (21 ± 0.46 ng • ml^{-1} in immature fish to 24.3 ± 0.7 ng • ml^{-1} in mature fish)

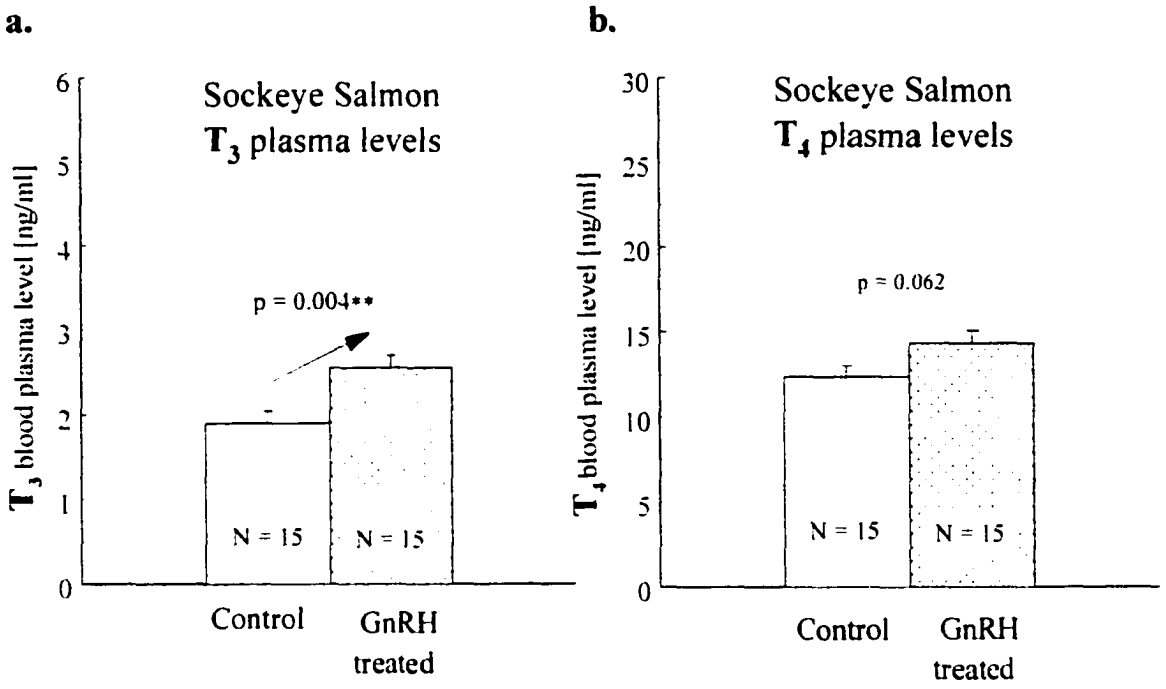


Figure 3.7.a & b.: T_3 (Figure 3.7.a) and T_4 plasma levels (Figure 3.7.b.) in two year old sockeye salmon compared between saline injected control (open bars) and GnRH injected (striped bars) sockeye salmon. The GnRH injection increased the T_3 plasma level significantly (ANOVA, $p = 0.004$) from 1.9 ng/ml to 2.56 ng/ml, while T_4 levels did not change significantly (control 12.39 ng/ml, GnRH treated 14.31ng/ml).

Deiodinase activity response to a GnRH challenge

Response of T_3 and T_4 plasma levels to a GnRH challenge in sockeye salmon

T_3 plasma levels increased significantly, following a GnRH challenge (Figure 3.7.a). T_4 levels however, did not seem to be significantly altered by the hormonal action of GnRH (Figure 3.7.b).

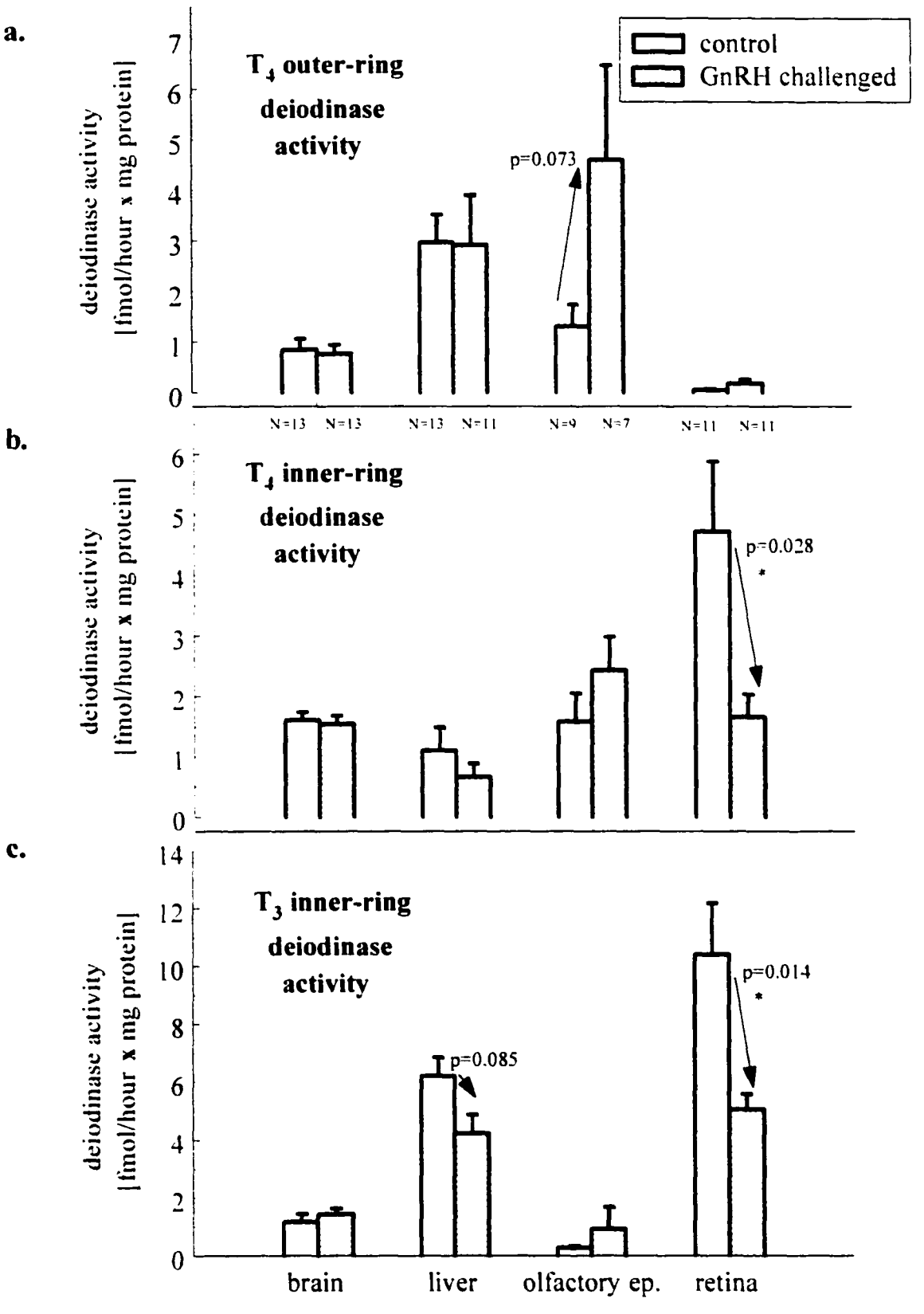


Figure 3.8.a, b & c (legend next page)

Figure 3.8. (legend): Figure 3.8. shows deiodinase activity in microsomal fractions from different tissues of three-year-old immature sockeye salmon. For each tissues the value that is represented by the open bar stands for deiodinase activity in control fish whereas the striped bar stands for deiodinase activity in GnRH challenged fish. T_4 outer-ring deiodinase (ORD) (Figure 3.8.a) activity did not change significantly corresponding to a GnRH challenge in either one of the tissues, but in the olfactory epithelium T_4ORD activity increased in a non-significant manner ($p=0.073$, ANOVA). T_4 inner-ring deiodinase (IRD) (Figure 3.8.b) activity did not change significantly in response to GnRH challenge in brain, liver and olfactory epithelium but decreased significantly ($p=0.028$, ANOVA) in the retina. T_3 IRD activity (Figure 3.8.c) did not change significantly in response to a GnRH challenge in brain, liver and olfactory epithelium but also decreased significantly in the retina ($p=0.014$, ANOVA).

T_4 outer-ring deiodinase (ORD) activity

In response to a GnRH challenge, T_4 outer-ring deiodinase activity did not change significantly in the microsomal fraction sampled from either one of the tissues (Figure 3.8.a.). However, in the olfactory epithelium the ORD increased in a non-significant manner ($p=0.073$, ANOVA) based upon the high standard error between the samples. T_4 ORD activity in the olfactory epithelium had not been described up to date.

T_4 inner-ring deiodinase (IRD) activity

Subsequent to GnRH treatment, T_4 IRD activity (Figure 3.8.b) did not change significantly in brain, liver and olfactory epithelium but decreased significantly ($p=0.028$, ANOVA) in the retina. Like T_4 ORD activity, T_4 IRD activity had not been monitored in the olfactory epithelium or the retina up to date.

T_3 IRD activity

T_3 IRD activity (Figure 3.8.c) did not change significantly in response to a GnRH challenge in brain, liver and olfactory epithelium but decreased significantly in the retina ($p=0.014$, ANOVA). Like the activity of the other deiodinases, T_3 IRD activity had not been discovered in the olfactory epithelium or the retina previous to this study.

In summary deiodinase enzymes were active in all of the tested tissues but activity was only significantly decreased by GnRH treatment with respect to IRD activity in the retina.

III. 4. Discussion

T_3 levels in juvenile sockeye salmon

T_3 levels in control fish were low and stable at all times of sampling and are typical for fish that are reared in a stimulus-poor hatchery environment (Dickhoff & Sullivan, 1987). Control fish for my behavioural experiments (Chapter II) came from the same treatment group and therefore it was verified that T_3 levels during the olfactory imprinting period (Chapter II) were low and stable in control fish. After 6 days into a 30-day challenge with T_4, T_3 levels were unchanged relative to controls. Only a challenge with T_3 or a combined T_3T_4 treatment raised the T_3 levels significantly. Based on these results, T_4 challenges are not suitable to increase the availability of the physiologically active T_3 (Cyr & Eales, 1996; Eales, 1995) and may even lead to an activity decrease of the enzyme that converts T_4 to T_3 in the liver. Thus, the suggestion of Eales and Brown (1993) that T_4 treatment can be counterproductive when one attempts to achieve an increase of T_3 availability in blood plasma is supported by my data from sockeye salmon. The fact that only the T_3T_4 treated fish in the behavioural experiments, conducted in Chapter II of this study, reliably imprinted, provides additional evidence that an increase in the availability of T_3 and T_4 can initiate processes that ultimately lead to olfactory imprinting.

T_4 levels in juvenile sockeye salmon

T_4 levels in the control group of juvenile sockeye salmon decreased between day 90 and 97 after the onset of PST. During PST, salmon in nature and to a lesser extent in a

hatchery environment undergo an increase in T_4 serum levels (Hoar, 1988), which terminates at the end of PST. The declining T_4 serum levels that occurred between day 90 and day 97 in my study suggest that samples were taken past the peak of PST. Morphological features like parr-mark loss, silvering and first scale loss (described in Chrisp & Bjorn, 1978), used as indicators for the PST in this study, therefore appear to be suitable indicators for the onset of the PST stage in sockeye salmon.

T_4 levels were not altered in correspondence with T_3 treatment but increased significantly in response to T_4 and a combined T_3T_4 treatment. Peak T_4 serum levels subsequent to T_4 and T_3T_4 treatment were within the range of naturally occurring peak values for T_4 in hatchery-reared and wild coho and Atlantic salmon around PST (Grau et al., 1981; Hasler & Scholz, 1983, Youngson & Simpson, 1984). Therefore, T_4 challenges are well suited to elevate the level of the physiologically inactive T_4 serum levels but fail to elevate the levels of the physiologically active T_3. T_4 is assumed to be less physiologically active than T_3 because T_3 has a much higher affinity to nuclear binding sites (Eales & Brown, 1993). Whether T_4 can bind to nuclear binding sites in target tissue cells at all is not clear, since nuclear T_4 receptors have yet to be described (Cyr & Eales, 1996). Moreover, T_4 challenges increase the hepatic inner-ring deiodination of T_4 to the phyiologically inactive r-T_3 and decrease the conversion from T_4 to the physiologically active T_3 (unpublished data from this study). The activation of those two pathways is normally correlated with an oversupply of T_4 and considered to be a mechanism of T_4 disposal.

Aside from monitoring thyroid hormone levels, the results obtained in this study verified that the T_3 and T_4 challenges in my behavioural experiments did not interfere

with natural peaks of THs, which was crucial for the interpretation of my behavioural results (Chapter II).

T_3 and T_4 blood plasma levels in adult sockeye salmon

From one to two weeks before maturation until maturation, T_3 serum concentrations stayed unchanged, whereas T_4 concentrations decreased significantly in wild sockeye salmon. T_4 and T_3 blood plasma levels are interpreted as indicators for different physiological states or processes.

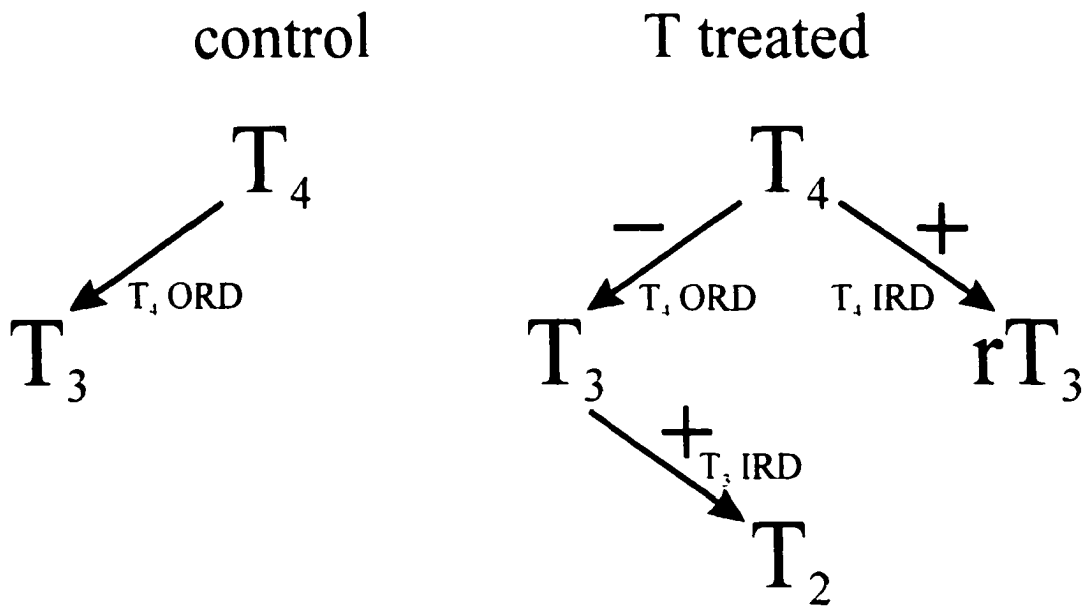


Figure 3.9: The influence of administration of thyroid hormones in feed on the pathways of T_4 hepatic deiodination in immature rainbow trout. –, inactivation of deiodinase pathway relative to controls; +, activation of deiodinase pathway relative to controls (combined from Eales & Brown, 1993 and Morin et al., 1995)

T_4 is primarily responsible for iodine supply and regulates energy balance in somatic growth and metabolism (Eales, 1979, Cyr and Eales, 1996). When salmon are homing, feeding ceases and energy investment is shifted away from somatic growth and towards migratory movement and gonadal growth. Near completion of the migration, energy supplies are depleted and T_4 blood plasma levels typically decline (McBride, 1967), which might explain the declining T_4 levels in the wild sockeye salmon that were approaching final maturity at the end of their migration in this study.

Under hatchery rearing conditions, no energy is invested into migratory behaviour and accordingly T_4 plasma concentration stayed at the same high level, when immature and mature fish were compared. The T_3 serum levels on the other hand, dropped significantly between immature and mature fish of the same age. The difference in the physiological state between hatchery-reared and wild sockeye salmon might be responsible for this phenomenon. My hatchery-reared sockeye salmon actively fed until final maturation and therefore did not undergo the typical energy depletion, observed before maturity in wild fish that can critically influence the thyroid hormone household (McBride, 1967; Eales, 1979; Cyr and Eales, 1996).

Despite the significant drop in plasma T_4 concentration in wild fish, their T_3 levels stayed fairly constant. Thus, lower T_4 production in the thyroid, which occurs at final maturation, may be compensated by an increase in T_4 to T_3 conversion in the liver. GnRH might initiate this regulation of deiodinase activity at maturity (this study).

When thyroid hormone levels were compared between immature and mature hatchery-reared fish of the same age, T_4 levels stayed constant and T_3 serum levels decreased significantly, which is in complete contrast to the results obtained from wild

fish. This T_3 drop may be caused by a decrease in outer-ring deiodinase activity leading to a lack of T_4 to T_3 conversion or alternatively an increase in T_3 inner-ring deiodinase activity, leading to increased T_3 disposal. Hatchery-rearing conditions, void of the sensory stimulation experienced in nature during the homing migration, can lead to a deficiency of sex steroids produced by the gonads during vitellogenesis in hatchery-reared fish (Donaldson et al., 1981). Sex steroids, in turn, can severely affect T_3 and T_4 serum levels (Cyr & Eales, 1996), which is consistent with my results. In conclusion, the experimental evidence gathered in this study, supports the assumption that the hormonal household of salmonids is severely affected by rearing conditions.

Deiodinase activity:

While outer-ring-deiodinase (ORD) activity has only recently been described in the retina of fish (Orozco et al., 2000), ORD in the olfactory epithelium and inner-ring deiodinase (IRD) activity in the olfactory epithelium and the retina is described for the first time in this study. IRD activity in the retina was very high, even in comparison to IRD activity in the liver, an organ that has a central role in the control of the thyroid hormone household (Eales, 1995). Therefore, T_3 availability within the retina might be altogether detached from fluctuations in T_4 or T_3 serum levels and the effect of exogenous T_4 challenges on this tissue may be highly dependent on endogenous deiodinase activity. In the olfactory epithelium ORD and IRD activity was very high (compared to liver), which suggests that the olfactory epithelium is also able to regulate its own T_3 availability. GnRH treatment increased the circulating T_3 serum level and did not affect the T_4 level. This result can be interpreted as a GnRH based stimulation of ORD activity.

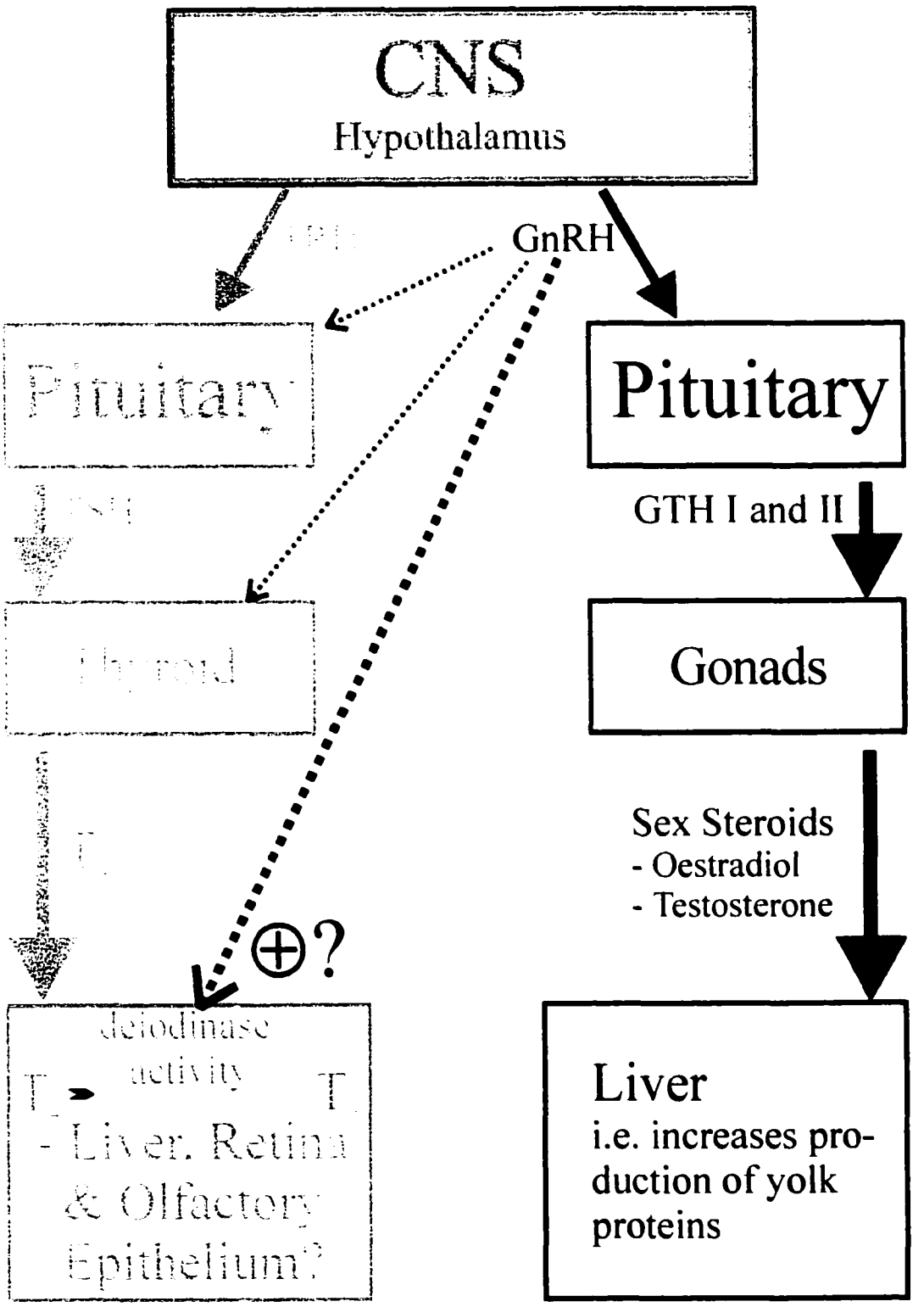


Figure 3.10.: A model for possible GnRH mediated interactions between the reproductive and the thyroid system.

However, since GnRH failed to initiate significant changes in ORD or IRD activity in the liver, the increased serum T_3 concentration may have been the result of increased ORD activity in an organ that was not investigated in this study. GnRH decreased IRD activity in the retina and did not initiate significant changes in ORD or IRD activity in liver, brain or olfactory epithelium. ORD activity in the olfactory epithelium however, appeared to be increased as well ($p=0.073$, ANOVA). Possibly, the high standard error within the GnRH treated group pushed the p-value above the significance level of $p = 0.05$, which might indicate that the GnRH treatment affected ORD activity in the olfactory epithelia of some fish while others were unaffected. Overall, GnRH may represent the factor that regulates the tissue-specific availability of thyroid hormones and thus tunes sensory tissues for the tasks of homing and maturation.

In conclusion, with the help of Dr.Jeff Eales and Bruce Adams I demonstrated that the retina and the olfactory epithelium can control their thyroid household on a peripheral level and that GnRH can directly influence the deiodinase activity in the retina and possibly in the olfactory epithelium. T_3 could therefore still act as a neuromodulator during times of low T_4 availability, as observed in wild maturing salmon T_3 (this study). I therefore suggest that the reproductive and the thyroid systems can interact at the level of sensory receptor tissues (Figure 3.10) and thus possibly prepare those tissues for the special tasks of homing, independent from blood plasma levels of thyroid hormones.

Since the thyroid and/or the GnRH systems are far from being fully understood or simple in nature, I am fully aware that the attempt to clarify the issue made in this discussion, represents one possible explanation among many others.

Chapter IV: Heart-rate-conditioning: a classical conditioning paradigm used to investigate hormonal influence on the olfactory system in sockeye salmon

IV.1.Introduction:

Knowledge about salmon homing is largely based on behavioural studies. A large number of behavioural studies have been undertaken since Hasler & Wisby (1951) conducted the first experiment that clearly demonstrated the dependence of precise homing on olfactory perception. In summary, it became obvious that orientation during the freshwater phase of homing was based on the recognition of olfactory stimuli internalized during juvenile imprinting. Hara et al. (1970) and Hasler, Scholz & Wisby (Hasler & Scholz, 1983) first used electrophysiology to further investigate neuronal processes underlying homing navigation. Electrophysiology provided quantitative and qualitative detail about the olfactory system but long-term studies entailing multiple testing of the same animal were impossible due to the invasive nature of electrophysiology. The need for a testing procedure that would allow for quantitative and qualitative data and multiple testing throughout ontogeny became obvious. Heart-rate-conditioning can elucidate threshold concentrations for the detection of odorants and offers the added possibility for multiple testing throughout different life stages. Hirsch (1977) first employed the technique for olfactory research in salmonids. In his experiments, the onset of an olfactory stimulant was followed by a mild electroshock while monitoring the heart rate. The natural heart-rate-reduction or bradycardia

experienced in response to the weak shock would shift in its occurrence from immediately following the shock in unconditioned fish, to immediately following the odorant and this way occurring before the shock in conditioned fish. Since fish were kept aerated, anaesthetized and immobilized during testing, they could be used repeatedly after a period of regeneration. Thus, it became possible to determine threshold concentrations for olfactory clues during ontogeny and to detect the influence of possible hormonal modulators of olfactory sensitivity in the same individual.

Heart-rate-conditioning is also based on the modulation of the myogenic heart pacemaker to reduce heart–rate in response to the unconditioned and conditioned stimulus. Whether the neuronal connection between the stimuli and the heart pace maker is established directly via a reflex arch or indirectly via the central nervous system is unclear. The technique it is not restricted to the olfactory system and has also been used to investigate spectral sensitivity of the retina (Beauchamp & Rowe, 1977, Hawryshyn & Beauchamp, 1985, Browman & Hawryshyn, 1992). This suggests that neuronal information may be processed in one of the multimodal centers of the central nervous system. Hence, heart-rate-conditioning is considered to be a neurophysiological as well as a behavioural methodology to determine olfactory sensitivity. Heart-rate-conditioning is highly dependent on the health of the tested fish because it is dependent on neuronal computation involving the whole nervous system over an extended period of time.

I used the technique to determine whether gonadotropin-releasing hormone (GnRH) can influence the threshold concentration to an imprinting odorant in sockeye salmon. Thyroid and gonadotropin hormones, alone or interactively, have been suggested to facilitate juvenile olfactory imprinting and odorant recognition in homing salmon

respectively (Figure 3.1)(Hasler & Scholz, 1983; Cyr & Eales, 1996). Possible interactions between the thyroid and the gonadotropin systems are described in detail in the Introduction of Chapter 3 in this dissertation.

In this study, heart-rate-conditioning experiments were used to investigate aspects of olfactory imprinting in the following way. First, immature two-year-old sockeye salmon were exposed to two odorants and both forms of thyroid hormone. Thus, I tried to achieve olfactory imprinting at a developmental stage that is temporally detached from the naturally occurring process of imprinting. Second, heart-rate conditioning tests were conducted to determine the threshold concentrations of the two potential imprinting odorants six months after odorant and hormone exposure. This way, thresholds to the two imprinting odorants were determined in immature sockeye salmon at a time when the fish are not hormonally tuned towards maturation or homing and imprinting odorant recognition. Third, the possible effect of GnRH treatment on the threshold concentrations to an imprinting odorant was determined in immature fish through heart-rate conditioning.

In summary, I tried to determine whether sensitization to an imprinting odorant during the imprinting and the odorant recognition process can be accomplished by using a certain odorant and hormone combination at a developmental stage that is temporally detached from the respective task in nature. This approach allowed for easier interpretation of possible hormonal influence than previous studies (Hasler & Scholz, 1983; Dittman et al., 1994; Nevitt et al., 1994; Dittman et al., 1997).

IV.2. Materials and methods

1. For general materials and methods regarding egg sampling, incubation, rearing, thyroid hormone feed treatment and GnRH treatment see materials and methods of Chapter II and III.

Timing of thyroid hormone feed treatment and odorant exposure

Food of the test fish was treated for 30 days with T_3 (12 ppm) and T_4 (120 ppm) six months before testing in the heart-rate-conditioning set-up (at two years of age). Fish were fed 1-2% of their average body weight daily. Parallel to the hormonal treatment, sockeye salmon were exposed to $MnCl_2$ and L-Alanine at a concentration of 10^{-6} M. Control groups were exposed to the odorants without additional hormone treatment.

Timing of GnRH treatment

In September of 1998 (age, three years) approximately 50% of the fish that were exposed to T_3, T_4, $MnCl_2$ and L-alanine matured. For GnRH treatment, completely silvery fish without any morphological signs of maturity (i.e. darker body colouration, humpback) were chosen. The immature fish were injected with 20 $\mu g \bullet kg^{-1}$ body weight of mammalian GnRH analogue (D-Ala-6 N-ethyl amide dissolved in 9‰ saline) into the expaxial musculature twice, 36 h and 12h before testing in the heart-rate-conditioning set-up.

Experimental set-up and procedure

The central component of the heart-rate-conditioning set-up (Figure 4.1.) was a fish holding tank with a slow, constant flow to transport odorants away from the nasal epithelium for discharge. A foam-padded, adjustable fish holder keeps the test fish in a position facing the inflow end of the tank. Oxygen saturation at the gill epithelium was maintained via a constant flow of one L • min^{-1} through a respirator tube inserted into the mouth of the fish. Heart rate was monitored through heartbeat electrodes (gold plated cast silver heart-beat electrodes, Grass, E6GH) touching the body surface behind the origin of the pectoral fins. Electrophysiological signals picked up by the electrodes were amplified by a pre-amplifier (World Precision Instruments, DAM 70) and further visualized by an oscilloscope (Tektronix Inc., Portland, Oregon, Type 502 A dual beam oscilloscope). The amplified trace was digitized (Labmaster A/D board, 12 bit, Scientific Solutions) and saved in a personal computer, facilitated by customized recording software (software designed in ASYST, Keithly ASYST Software). Odorants were administered through a glass tube (5 mm inside diameter) positioned 2 mm from the inflow end of the nares. Water flow through the glass tubing was controlled by a peristaltic pump (Cole-Parmer, Masterflex, Model No. 7015) and kept constant at 20 ml • min^{-1}. To administer the odorants, the flow of background water was stopped and immediately replaced by flow out of an odorant tank through computer controlled three-way solenoid valves (Burkert Contromatic Corp., 3-way Type 300). The use of a peristaltic pump ensured that pressure differences during the switch from the background water to the odorant were kept minimal.

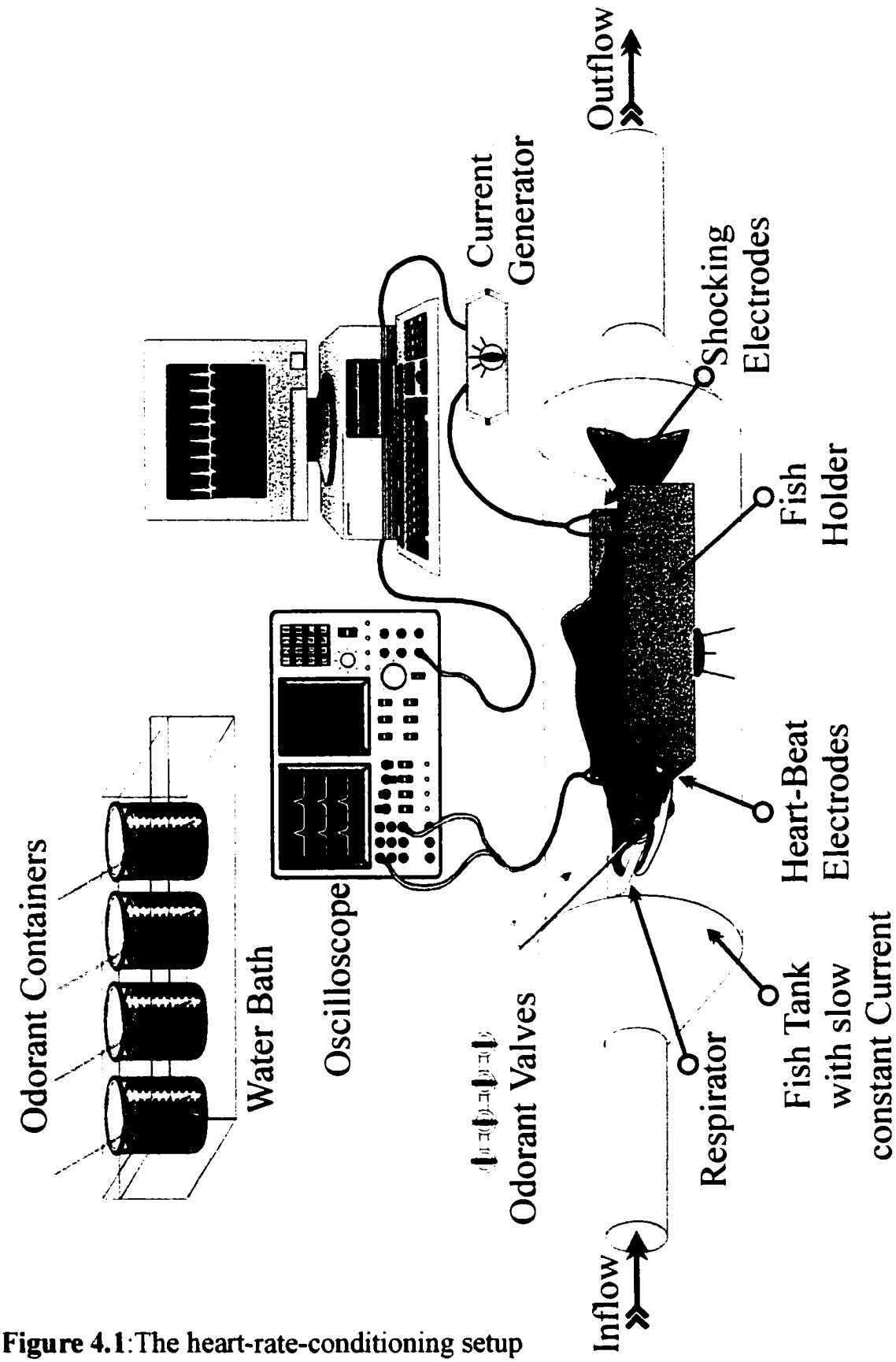


Figure 4.1:The heart-rate-conditioning setup

Shocking electrodes were positioned on both sides of the caudal peduncle and shock timing (500ms-1s) and intensity (5-25 mA, AC) were adjusted to induce consistent bradycardia during the conditioning process.

In preparation for an experiment, fish were transported to the experimental set-up (2 minutes) and anaesthetized in MS222 (tricaine methanosulfonate, concentration in water bath 0.1 g • L^{-1}). Once completely anaesthetized, fish were injected with the muscle relaxant Flaxedil (gallamine triethiodide, 10mg • kg body $weight^{-1}$) and fitted into the foam lined fish holder where aspiration was started immediately. Throughout the experiment, fish were maintained under mild anesthesia (3 mg • kg body $weight^{-1}$ intramuscular injection of Marinil = Metomidate hydrochloride). Heartbeat and shocking electrodes were tucked under the pectoral fins and positioned at the caudal peduncle respectively, before fish were left for 30 minutes to acclimatize. During this period heartbeat was monitored and typically stabilized between 80-100 beats per minute. Following acclimatization, conditioning was started by presenting a two-second pulse of an odorant at super threshold concentration, followed by a one-second shock. The shock intensity had to be adjusted to reliably initiate a bradycardia since only fish that displayed a bradycardia to the shock could be conditioned to the odorant. The set-up was calibrated temporally using methylene blue dye to determine when the highest odorant concentration reached the nasal epithelium (Figure 4.3.). The onset of the shock was timed beginning two seconds after the highest odorant concentration at each valve. Fish were stimulated with five concentrations from $10^{-7.5}$ to 10^{-2} M of L-alanine and $MnCl_2$ (ten odorant channels in total). Each testing session consisted of up to 50 trials, by which time a test fish had to be conditioned or was dismissed (30% of all fish). The duration of

time between individual trials within a session was chosen randomly between 30 and 150 seconds. During the entire session a masking valve was operated at a random frequency: without this precautionary measure, fish would become conditioned to the noise of opening and closing valves instead of the odorant in test trials.

The relative cardiac deceleration was calculated by dividing the largest interbeat interval during the ten seconds of odorant stimulation with the largest interbeat interval during the ten seconds before odorant stimulation. A criterion response was defined when a single interbeat interval during the odorant stimulation period was at least 1.04 times longer than the longest interbeat interval during the pre-stimulus period. The pre-stimulus interbeat interval mean and its standard deviation were calculated for each trial to allow for an evaluation and possible adjustment of the criterion level. For every 1% increase in the standard deviation of the pre-stimulus interbeat interval, the conditioning criterion was increased by 0.05 to ensure statistical robustness of the procedure. A fish was considered to be conditioned when it gave three consecutive criterion responses. The following ten blank trials served to detect possible conditioning to unknown extraneous stimuli other than the odorant.

Threshold determination began one hour after the last conditioning trial. During threshold determination, fish were shocked only when they showed a criterion response. Starting at $10^{-7.5}$ M, concentration was increased by 1-1.5 log units every two trials, until fish showed two consecutive criterion responses, at which time threshold intensity was recorded. To avoid false positives the threshold detection procedure would be repeated two more times.

After the experiment, fish were allowed to recover in a respirating tank overnight and were transferred back into their holding tank next morning.

Set-up calibration

To obtain absolute, rather than relative threshold concentrations and to ensure that the highest odorant concentration was immediately followed by the shock, the timing of the odorant arrival at the naris had to be precisely calibrated. In the first calibration step, I used ten known standard concentrations of methylene blue, determined their absorption at a wavelength of λ = 688 nm in a spectrophotometer (Nova Spec II) and calculated a calibration curve that related concentration to absorption. Then one of the odorant containers was filled with a known concentration of methylene blue (10^{-3} M) and I monitored the period of time from opening of the solenoid valve until the dye reached the outflow end of the odorant supply tube. From that point forward, the dye solution was collected for the next 70 seconds, sampling every two seconds. Thus, the timing and concentrations of the testing odorants at the end of the odorant supply tube was monitored for channel one, five and eight. Three channels were monitored since the solution had to travel a variable pathlength, depending on the channel that was used.

IV. 3. Results:

Set-up calibration

Figure 4.2. shows the calibration curve that related known dye concentrations to their absorption in a spectrophotometer.

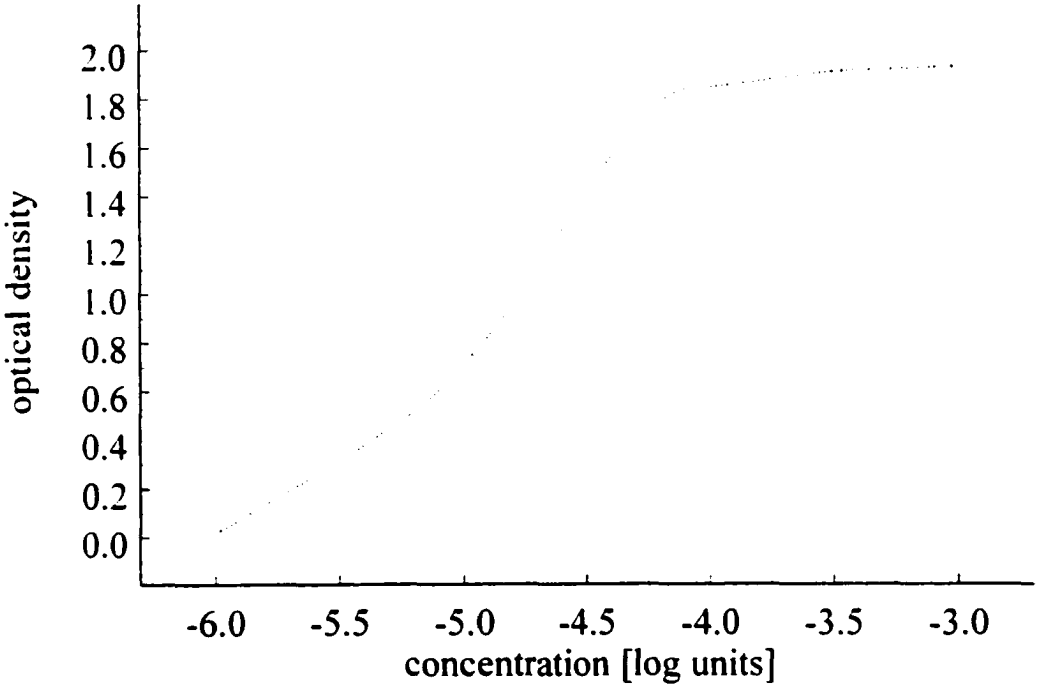


Figure 4.2.: Calibration curve for methylene blue. Absorption of light (optical density) at a wavelength of λ = 688 nm in the Nova Spec II was related to the known concentrations of methylene blue.

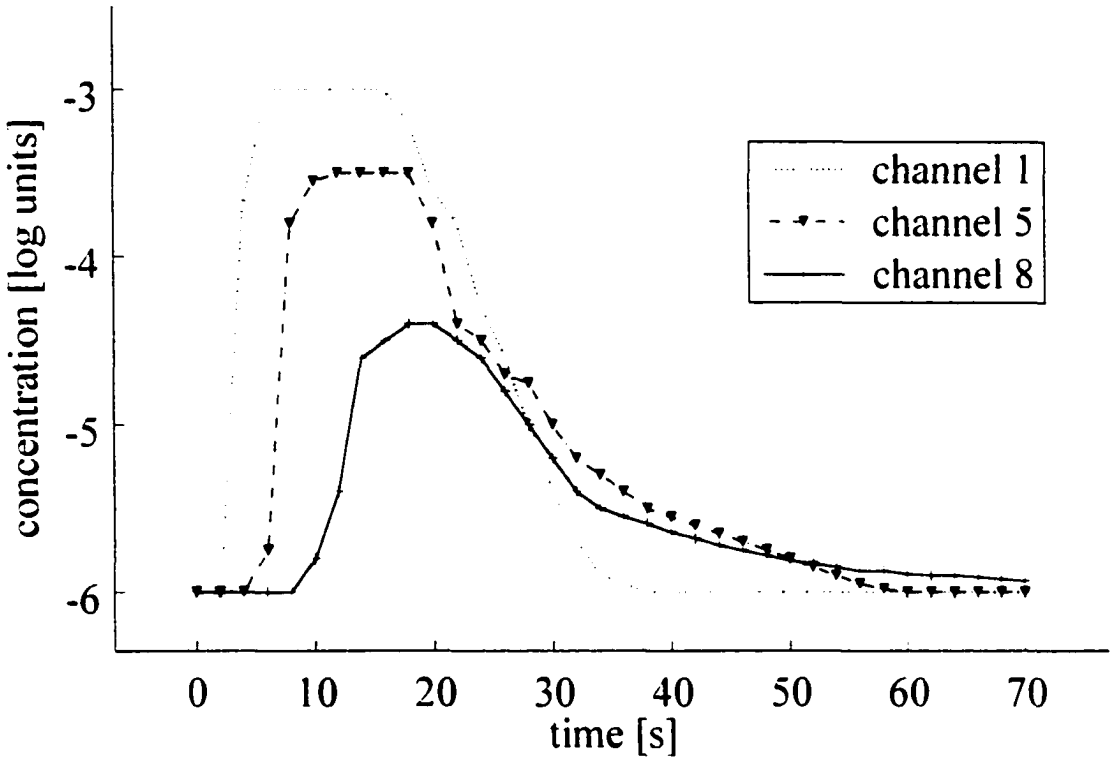


Figure 4.3.: Methylene concentration over time depending on the channels that were used. For all tests the odorants were running out of a container that was filled with a methylene blue concentration of 10^{-3} M. In channel 1 (open circles connected by dotted line) the maximum concentration of 10^{-3} M was reached after six seconds and started to drop back to the starting concentration 10 seconds later. In channel 5 (black triangles connected by stippled line) the odorant concentration rose in 12 seconds to $10^{-3.5}$ M, half a log unit lower than in the odorant container, stayed at that concentration for eight seconds until it gradually dropped back to the starting concentration within 40 seconds. The maximum concentration of $10^{-4.4}$ M that was reached in channel 8 (black crosses connected with solid line), was 1.4 log units below the known odorant tank concentration. It was reached after 18 seconds, stayed at this level for two seconds and slowly decreased over the next 48 seconds.

In a second calibration step, it was determined when the highest dye concentration could be monitored at the outflow end of the odorant delivery tube. For all tests, the odorants were running out of a container that was filled with a methylene blue concentration of

10^{-3} M. In channel 1 (open circles connected by dotted line) the maximum concentration of 10^{-3} M was reached after six seconds and started to drop back to the starting concentration ten seconds later. In channel 5 (black triangles connected by stippled line), the odorant concentration rose in 12 seconds to $10^{-3.5}$ M, half a log unit lower than in the odorant container, and stayed at that concentration for eight seconds until it gradually dropped back. The maximum observed concentration of $10^{-4.4}$ M in channel 8 (black crosses connected with solid line) was 1.4 log units below the known odorant tank concentration. It was reached in 18 seconds, stayed at this level for two seconds and slowly decreased over the next 48 seconds, but never completely dropped back to the starting level. The calibration allowed me to deliver the highest odorant concentration two seconds before the shock in all channels. It also revealed the odorant profile over time that could be expected in each odorant channel.

Trials to criterion acquisition

Only seven out of 23 or 30.4 % of the fish were conditioned to the odorant within the first 21 trials (Figure 4.4.a, column 1 and 2). For 16 out of 23 or 69.6 % of the fish, it took between 21 and 60 trials until bradycardia could be detected in response to the odorant (Figure 4.4.a, column 3-6).

Figure 4.4.a.

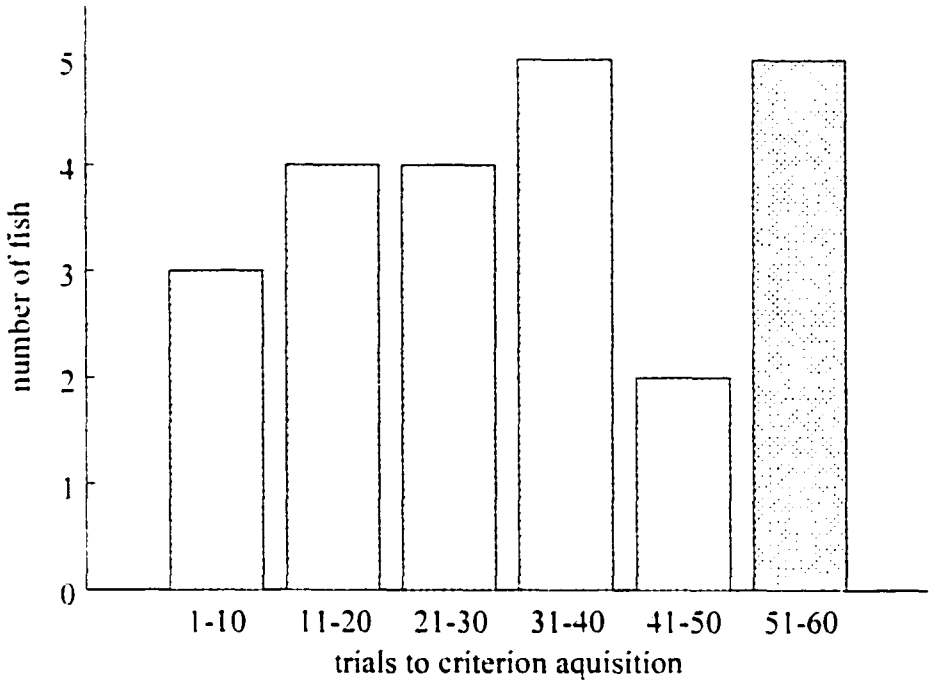


Figure 4.4.b.

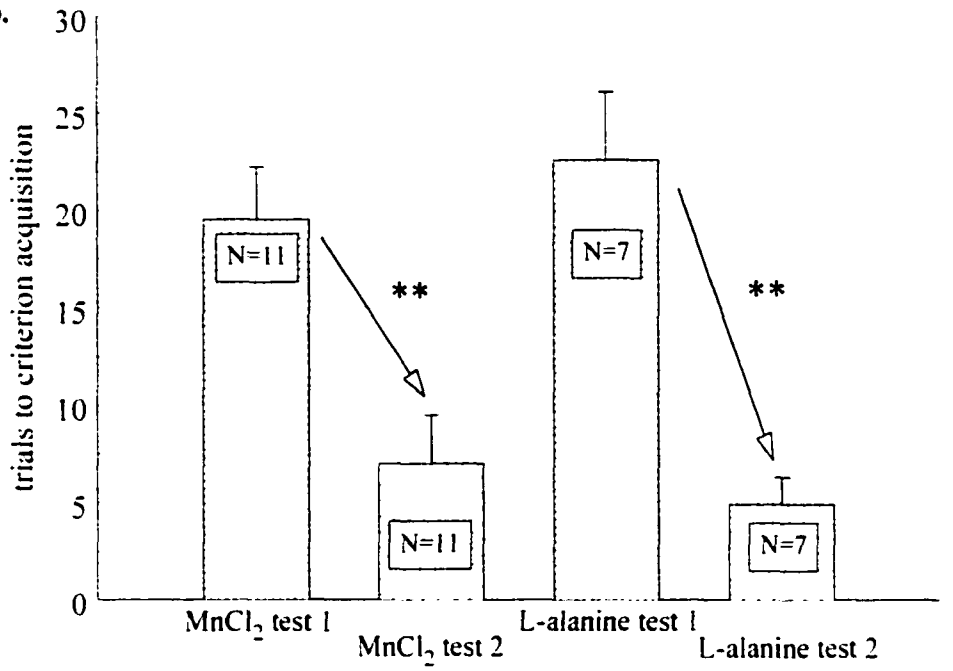


Figure 4.4.a. & b.: (legend next page)

Figure 4.4.a. & b: Figure 4.4.a. shows the number of trials to criterion acquisition for sockeye salmon in the heart-rate-conditioning set-up for a total of 23 fish. All fish were exposed to $MnCl_2$ and L-alanine for thirty days, six months before the experiment. Three fish acquired criterion in 1-10 trials, four in 11-20 trials, four in 21-30 trials, five in 31-40 trials, two in 41-50 trials and five in 51-60 trials. The experiment was terminated if the fish could not be conditioned after 60 trials.
In Figure 4.4.b. I compared the number of trials until criterion acquisition between test one (tight pattern) and test two (loose pattern) when using $MnCl_2$ or L-alanine as test odorants. The number of trials until criterion acquisition decreased significantly for both odorants from test one to test two (paired t-test, $MnCl_2$ $p = 0.004$, L-alanine $p = 0.003$). A significant difference in trials until criterion between the odorants could neither be found in test one nor in test two (independent samples t-test, test one $p = 0.493$, test two $p = 0.546$).

However, the number of trials until criterion decreased significantly for both odorants from test one to test two (paired t-test, $MnCl_2$ $p = 0.004$, L-alanine $p = 0.003$) (Figure 4.4.b).

Possible effects of thyroid hormones for imprinting and GnRH for odorant recognition

To determine the possible effect of a combined T_3T_4 challenge, parallel to odorant exposure at two years of age, results from this cohort were compared with results obtained from fish that were exposed to the odorants without the hormonal challenge. No significant difference in the threshold concentration for the imprinting odorants $MnCl_2$ and L-alanine could be detected between the two groups, six months after exposure in immature fish (Figure 4.5.a. and 4.5.b. column one and two).

Figure 4.5.a.

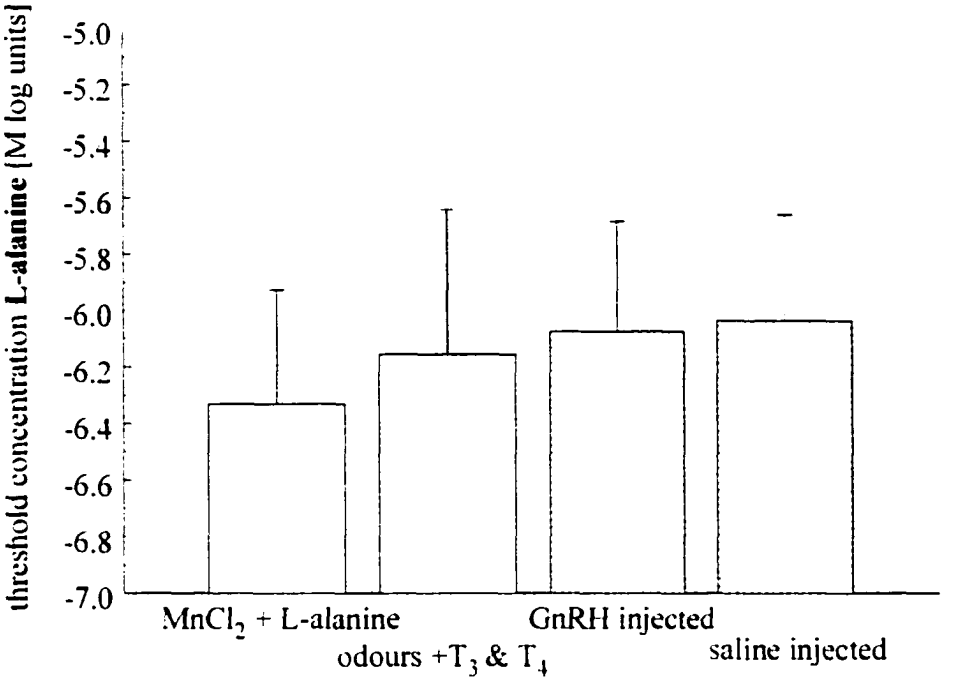


Figure 4.5.b.

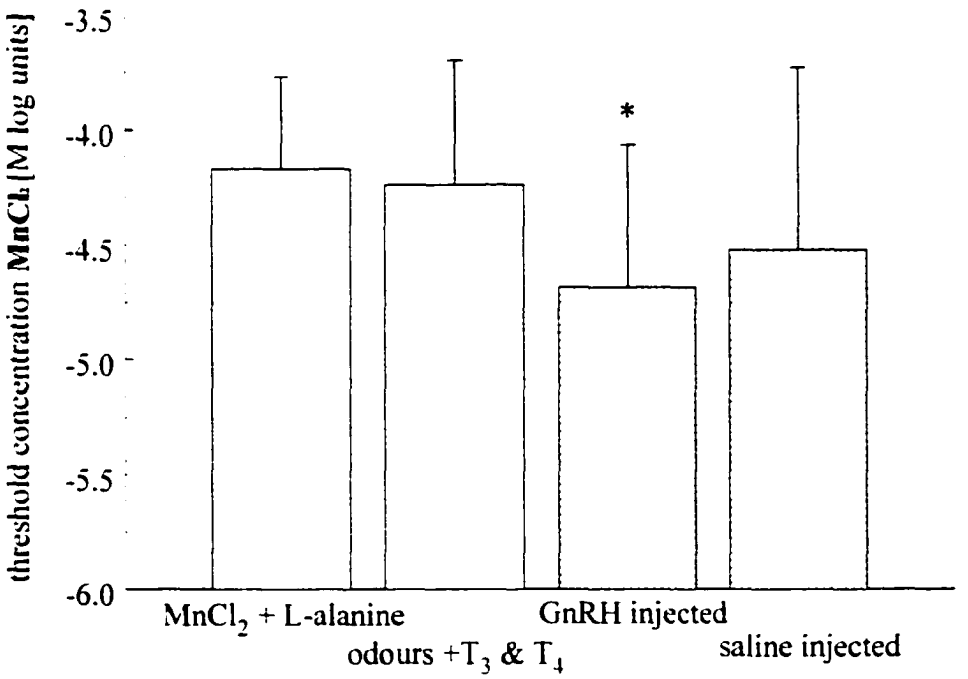


Figure 4.5.a. & b.: (legend following page)

Figure 4.5.a & b: Figure 4.5.a. shows the threshold concentration of two and a half year old immature sockeye salmon for the detection of L-alanine. Threshold concentrations for L-alanine for fish that were exposed (six months prior to testing) to L-alanine and $MnCl_2$ ($10^{-6.33} \pm 10^{-0.4}$ M, column 1) did not differ (independent samples t-test) from fish that were additionally exposed to thyroid hormones during the imprinting period ($10^{-6.16} \pm 10^{-0.51}$ M, column 2). Neither GnRH injections nor saline injections before testing altered the threshold concentrations for L-alanine (paired t-test) (GnRH injected: $10^{-6.08} \pm 10^{-0.38}$ M, column 3; saline injected: $10^{-6.04} \pm 10^{-0.39}$ M, column 4).
In Figure 4.5.b. the threshold concentrations to $MnCl_2$ for sockeye salmon that were, treated under the same protocol as the fish in Figure 4.5.a., are shown. The major difference between the response to L-alanine and $MnCl_2$ can be found in column 3. The GnRH injection in this case led to a significant (paired t-test, $p = 0.037$) decrease in the threshold concentration to $MnCl_2$ (from $10^{-4.24} \pm 10^{-0.54}$ M column 2 to $10^{-4.69} \pm 10^{-0.62}$ M, column 3). Control fish, injected with saline, did not display a significant change in their threshold concentration to $MnCl_2$ ($10^{-4.52} \pm 10^{-0.79}$ M, column 4).

When the same fish were injected with a 9 ‰ saline-GnRH solution, the threshold concentration for $MnCl_2$ decreased significantly (paired t-test, $p = 0.037$), while it did not change significantly in the control fish injected with saline. In contrast, the threshold concentration for L-alanine did not change significantly in response to a GnRH or saline injection (Figures 4.5.a. L-alanine & b. $MnCl_2$, column 3 for GnRH, column 4 for control fish).

GnRH therefore decreased the threshold concentration to $MnCl_2$ but did not alter the threshold concentration to L-alanine even though the fish had experienced exposure to both odorants plus thyroid hormones six months prior to testing.

IV. 4. Discussion:

A low number of trials to criterion acquisition has been suggested as an indicator for a sensitive period for olfactory imprinting (Morin et al., 1989 a & b). In the same study the period of memory retention of olfactory stimuli learned during this sensitive period was presumably increased. I therefore assumed that the number of trials to criterion acquisition would decrease when the fish had previously been imprinted to this odorant. This assumption could not be confirmed by my data. The number of trials to criterion did not decrease when fish had previously been exposed to the odorants and thyroid hormones. Nevertheless, a significant threshold decrease to $MnCl_2$ following two GnRH injections was taken to indicate successful imprinting to the odorant and represented the main result of this experiment. Therefore, odorant recognition based on long-term retention of olfactory information was not evident in faster conditioning but rather in a decreased threshold concentration to the imprinting odorant caused by GnRH. GnRH has been suggested as a neuromodulator in ORNs of the mudpuppy (*Necturus maculosus*) (Eisthen et al., 2000) consistent with the findings of Okubo et al. (2000) who discovered GnRH binding sites in the olfactory epithelium of the Japanese eel (*Anguillus japonicus*). Neuromodulation in the mudpuppy led to an increase in the magnitude of in inward current into single ORNs and enhanced their excitability. The reduced resting potential that has to be overcome to depolarize GnRH modulated ORNs could lower threshold concentrations to an odorant, since fewer odorant molecules must bind to receptor sites of odorant binding proteins to initiate depolarization. GnRH could also modulate deiodinase activity in the ORNs (Chapter 3) and thus increase the availability of

T_3, which has a nuclear binding site in ORNs (Cyr & Eales, 1996). It would be interesting to repeat the experiments conducted by Eisthen et al. (2000) in combination with a deiodinase blocker or different T_4 concentrations in the surrounding medium to clarify whether GnRH acts directly or indirectly by way of the deiodinase activation.

During the experiments conducted in this study certain details of the heart-rate conditioning experiments proved to be crucial to obtain reliable results. First, it was necessary to control precisely the delay between peak odorant concentration and the mild shock. The number of trials to criterion acquisition increased drastically in preliminary trials when the peak odorant concentration was not presented exactly two seconds before the shock. Moreover, it proved to be essential to elicit a strong and clearly detectable bradycardia in response to the shock, to warrant a conditioned response to the odorant later on. Second, a flexible threshold multiplier that was adjusted dependent on the regularity of heart beat, considerably increased the number of fish that could be conditioned. At the same time, a statistical power test estimated that the sliding scale multiplier would not produce more than ten false positives in 500 trials. Other authors had similar experiences. Morin et al. (1987, 1989 a & b) and Browman & Hawryshyn (1992) had to lower their threshold multipliers when working with olfactory stimuli in Atlantic salmon and visual stimuli in rainbow trout, respectively. Interbeat interval changes of 1.5 times, common in response to visual stimuli in heart-rate-conditioning experiments with goldfish (Hawryshyn, 1991), occurred in two out of 5000 trials that were conducted on more than 100 sockeye salmon and rainbow trout during 12 months of intensive testing in this study.

The high number of trials to criterion acquisition observed in this study was possibly the result of the nature of olfactory clues when compared with visual clues. The onset of visual stimuli can be accomplished within microseconds in comparison to seconds in olfactory stimuli. Therefore it is easier to achieve a perfect temporal sequence of stimulus and shock with visual stimuli.

The second result of my experiments was the difference in sensitivity changes to the two odorants, L-alanine and $MnCl_2$. Both odorants could be detected but once injected with GnRH, fish were hormonally tuned towards maturation and only the sensitivity towards $MnCl_2$ increased in response. Possibly this phenomenon can be explained by tank rearing conditions, where L-alanine, emitted from fish and fish food, is constantly present, and might have masked the amount of L-alanine that was added. Therefore, $MnCl_2$ is a likely candidate for the list of potential imprinting odorants in nature, along with $CaCl_2$, another bivalent cation suggested by Bodznick (1978a, 1978b). $MnCl_2$ was found in the natal stream of my test fish at concentrations similar to the ones used in my experiments and since it is washed out of rocks and stones, it is naturally abundant in fresh water (Clasen, 1998). The presence of $MnCl_2$ is not dependent on the residence of juvenile fish of the same population in the natal stream (Nordeng, 1971) and it is more stable in its concentration than organic compounds. A future study, in which behavioural changes to natal stream water would be recorded in response to substitution or extraction of single components of the stream water, would allow for the determination of odorants that are used for homing in nature.

Among the many questions about the olfactory imprinting process that remain unanswered one stands out from my point of view. How can the neuromodulatory action

of GnRH be selective to ORNs that are binding imprinting odorants? The heightened sensitivity of the guanylyl cyclase system to an olfactory imprinting odorant (Dittman et al., 1997) in coho salmon ORNs is suggestive of an explanation but depends on the turnover rate of ORNs after imprinting. Until we know whether ORNs constitute a static or dynamically phasic population of sensory receptor cells, I believe it will be impossible to explain the temporal and spatial selectivity of hormonal sensitization of ORNs within the olfactory epithelium

Overall, I believe that heart-rate conditioning experiments with more concentration steps and therefore a higher resolution can add new information to our knowledge of hormonal effects on the imprinting process. Concentration steps of one log unit, used in this study, are very coarse and likely mask small-scale threshold changes.

Chapter V: Electrophysiological assessment of peripheral olfactory sensitivity to non-imprinted and imprinted odorants

V.1.Introduction

In this study, electrophysiological recordings from the olfactory epithelium (Electro-Olfacto-Grams or EOGs) of sockeye salmon were used to assess peripheral sensitivity changes of the olfactory system in the context of olfactory imprinting and its hormonal background conditions. EOG responses to three odorants were recorded and subsequently it was monitored whether the onset of maturity, hormonal treatment (GnRH, T_3, T_4) or extended juvenile exposure to an odorant (imprinting) could modify the EOG. EOGs and electrophysiological recordings from the olfactory bulb (Electro-Encephalo-Grams or EEGs) have been used extensively to investigate neuronal mechanisms of olfactory imprinting (Cooper & Hasler, 1973, 1974, 1976; Hasler & Scholz, 1983; Hara, 1974; Cooper, Hara & Brown, 1982; Stabell, 1984, 1992). EOGs represent the summed receptor potentials of a large population of olfactory receptor neurons (ORNs). They are initiated when odorant molecules bind with specific receptors located on the cilia of the sensory neurons (Ottoson, 1971).

By recording EOGs, I monitored the initial step of olfactory perception that ultimately leads to olfactory imprinting. For the period starting with juvenile perception of odorants within the natal stream and ending with use of these odorants by maturing fish as orientational cues in the homing migration, odorant information concerning the natal stream has to be internalized. Where and how this odorant information is internalized in teleosts is unknown. In higher vertebrates, memory storage is commonly related to the hippocampus and amygdala of the CNS (Cahill & McGaugh, 1998; Paulsen

& Moser, 1998; Wallenstein et al., 1998). In contrast, Burton et al. (2000) pointed out that damage to the hippocampus or the amygdala did not impair learning or memory retention in an olfactory memory task. In the same study, however, the important role of the hippocampus for spatial memory tasks was confirmed. Therefore, olfactory information may be stored outside the classical spatial memory centers. In teleost fish, memory storage was long believed to be facilitated by the telencephalon (Overmier & Hollis, 1983), which comprises the structures suggested to be homologous to the hippocampus and the amygdala. Yet, ablation of the telencephalon did not impair memory in the goldfish (*Carassius auratus*) suggesting that another neuronal center must facilitate associative learning and memory storage (Overmier and Hollis, 1983). Although there seems to be a reluctance to accept such findings, they nevertheless suggest that memory storage can occur within unconventional memory centers in mammals and teleosts. In the context of olfactory imprinting, the sensitization of olfactory receptor neurons (ORNs), has been suggested as a means of internalization of stimuli. Nevitt & Moody (1992) and Nevitt et al. (1994) used single cell patch clamp recording techniques to demonstrate that isolated ORNs show higher levels of activity to an odorant if fish had been exposed to the odorant six months prior to testing. Dittman et al. (1997) added further evidence for peripheral olfactory sensitization during imprinting, by demonstrating increased guanylyl cyclase activity in ORNs of homing and mature salmon in response to an odorant previously used for imprinting. High guanylyl cyclase activity increases the availability of c-GMP in the cilia of ORNs (Breer et al., 1992). c-GMP in turn, can bind to cyclic nucleotide-gated ion channels to cause depolarization of ORNs in response to odorant molecules (Nakamura & Gold, 1987). In the context of olfactory

imprinting, the increased availability of c-GMP might reduce the negative potential that is maintained over the membrane of ORNs and reduce the number of stimulant molecules necessary to trigger depolarization. If this peripheral sensitization is restricted to ORNs that bind the imprinting odorant, it may represent the mechanism for olfactory imprinting. Nevitt and Dittman (1998) hypothesized that thyroid hormones might facilitate the imprinting process directly by a neuromodulatory effect on ORNs or indirectly by promoting the turnover of non-stimulated ORNs. In general, thyroid hormones certainly have the potential to re-model the central nervous system, a well documented phenomenon during amphibian metamorphosis (Denver, 1998), but many questions remain unanswered. How can the sensitized state of imprinted ORNs be maintained over the multi-annual oceanic phase in a salmon's life cycle when ORNs are the only sensory receptor cells that are continually turned over during adult life (Farbman, 1994)? Nevertheless, if the sensitized state of imprinted ORNs can cause permanent synaptic modification in higher centers of the olfactory system that is relayed back to newly emerging ORNs, memory storage might be independent from the turnover of ORNs. In this case, EOGs would be suitable to demonstrate imprinting related sensitization of the receptor cells in the olfactory epithelium.

I therefore chose EOG recordings as the methodology to investigate possible peripheral sensitization to olfactory stimuli in the context of olfactory imprinting. The research was conducted according to five steps. In step one, I determined general EOG responses to the three odorants that were chosen for my imprinting studies (the rationale for my choice of odorants was given in the introduction to Chapter II) in three-year-old immature sockeye salmon.

In step two, EOG responses from three-year-old mature fish were recorded and compared to results from the immature fish. Thus, I monitored whether the state of maturity could have a general or an odorant specific effect. In the third step, immature fish were treated with GnRH before electrophysiological recordings were undertaken. In this way, it was possible to compare the effects of maturity and GnRH on peripheral olfactory perception. My assumption of GnRH influence was based on three facts. Firstly, the homing migration and increased production and circulation of GnRH co-occur. Secondly, GnRH accelerates homing in sockeye salmon (Sato et al., 1997) and thirdly, GnRH can bind to ORNs (Okubo et al., 2000) and modulate their sensitivity (Eisthen et al., 2000).

In the fourth part of the study, fish were challenged with thyroid hormones, which have been, like GnRH, suggested as modulators of olfactory sensitivity in salmon (Scholz, 1980, Nevitt & Dittman, 1998). Subsequent to the treatment with both forms of thyroid hormones (T_3 and T_4), EOGs were recorded in three-year-old immature fish.

In experiments one to four, I established the influence of maturity, GnRH and thyroid hormones on peripheral olfactory sensitivity in fish that had never been exposed to an artificially increased concentration of an odorant and were therefore considered as being naive or not imprinted. In the fifth and last set of experiments, three-year-old fish that came from a cohort that had been exposed to $MnCl_2$ and T_3T_4 shortly after the PST, were tested. Since members of this cohort responded strongly to $MnCl_2$ in behavioural tests (Chapter II), they were considered imprinted to $MnCl_2$. I thus hoped to mimic the complete olfactory imprinting and odorant recognition cycle that salmon undergo in nature. EOGs in the imprinted fish were recorded in immature and mature fish to

determine the effect of maturity. Additionally, results from imprinted fish could be compared to non-imprinted fish at the same physiological stage, to answer the following question. Does imprinting change the peripheral neuronal sensitivity to the imprinting odorant?

V. 2. Chapter 5: Materials and Methods

1. For general materials and methods like egg sampling, incubation, rearing, juvenile odorant and thyroid exposure methodology and GnRH treatment see materials and methods of Chapter II.

Experimental set-up and procedure

Set-up: The central component of the electrophysiological recording rig (Figure 5.1.) was a fish holding tank with a slow, constant flow to transport odorant substances away from the nasal epithelium for discharge. The foam-padded adjustable fish holder fixed test fish in a position facing the inflow end of the tank. Oxygen saturation at the gill epithelium was maintained via a waterflow of 1 L • min^{-1} through a respirator tube that was inserted into the mouth of the test fish. Odorants were administered through a glass tube (5 mm inside diameter) pointing directly (distance 1 mm) at the surgically exposed nasal sac. The distance and the angle between the glass tube and the exposed olfactory epithelium were kept constant between individual fish. The flow rate through the glass tube was controlled by a peristaltic pump (Cole-Parmer, Masterflex, Model No. 7015) and kept constant at 20 mL • min^{-1}. To administer an odorant, the flow of background water was replaced for ten seconds by the flow out of an odorant tank through computer-controlled three-way solenoid valves (Burkert Contromatic Corp., 3-way Type 300). The use of a peristaltic pump ensured a constant water flow and buffered possible pressure differences during the switch from the background water to the odorant.

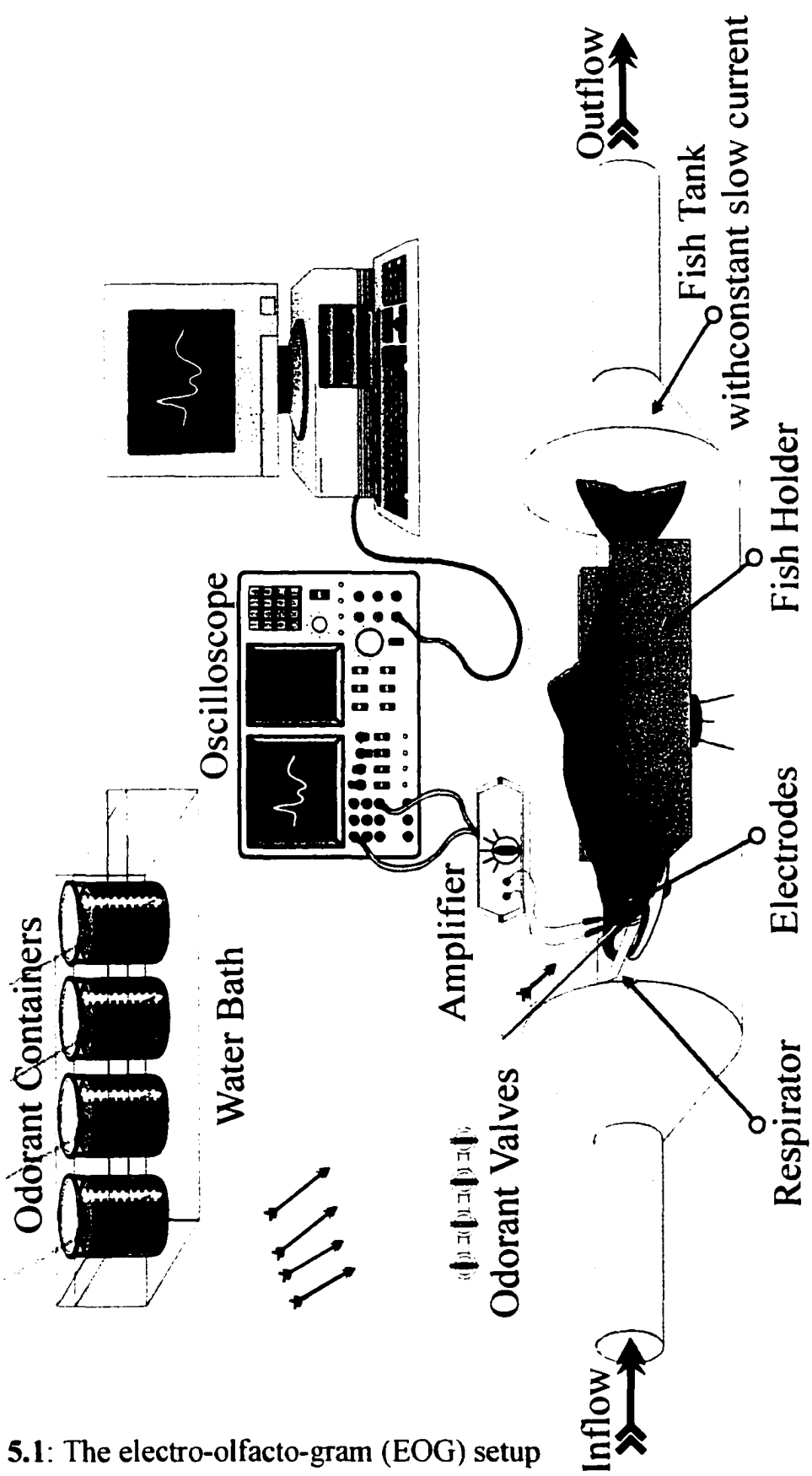


Figure 5.1: The electro-olfacto-gram (EOG) setup

EOG recording: Electrophysiological signals were recorded by Teflon® coated silver chloride electrodes (AM-Systems Incorporated, Order No. 7870, diameter = 0.03 mm), that were connected directly to a pre-amplifier (World Precision Instruments, DAM 70, Settings: high frequency cutoff filter = 1 KHz, low frequency cutoff filter = 0.1 Hz, amplification = 1000 times at the alternate current or AC setting) and displayed by an oscilloscope (Tektronix Inc., Portland, Oregon, Type 502 A dual beam oscilloscope).

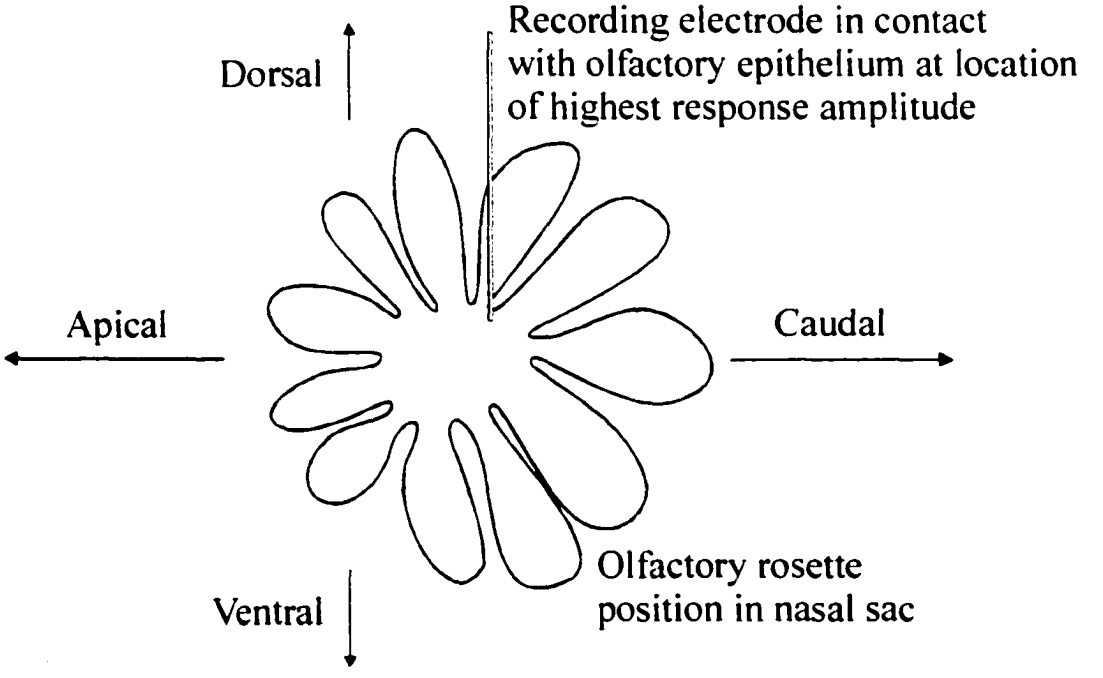


Figure 5.2.: The position of the recording electrode in the exposed olfactory epithelium. The recording position was chosen in extensive pre-testing sessions and provided the strongest possible response in all fish that were tested. It was located at the caudal-dorsal area of the flat part of the olfactory rosette, close to the longest raphae and possibly right in the center of the underlying olfactory nerve (Hara & Zhang, 1998).

In extensive pre-testing sessions a recording position (Figure 5.2.) was chosen that provided the strongest possible EOG response. It was located at the caudal-dorsal area of the flat part of the olfactory rosette, close to the longest raphae, possibly right in the center of the underlying olfactory nerve. The amplified trace was digitized (Labmaster A/D board, 12 bit, Scientific Solutions) and stored on a personal computer, using customized recording and analysis software (software designed in ASYST, Keithly ASYST Software).

To monitor the overall condition of the fish, heart rate was controlled during the acclimatization period at the beginning of each experiment. The heart-beat electrodes (gold plated cast silver heart-beat electrodes, Grass, E6GH) were positioned between the origin of the pelvic fins and the ventral body surface.

Experimental procedure for EOG recordings

In preparation for an experiment, fish were anaesthetized with MS222 (tricaine methanosulfonate, concentration in water bath 0.1 g • l^{-1}) while transported in buckets for two minutes from the holding facility to the laboratory. Completely anaesthetized fish were injected with the muscle relaxant Flaxedil (gallamine triethiodide, 10mg • kg body weight^{-1}), fitted into the foam lined fish holder and respiration (flow rate 1L • min) was started immediately. Throughout the experiment, fish were maintained under mild anesthesia by an intramuscular injection of Marinil (3 mg • kg body weight^{-1}, Marinil = Metomidate hydrochloride). Fish were left undisturbed for 30 minutes to acclimatize to the apparatus before electrophysiological recording began. During this period, heartbeat was monitored and typically stabilized between 80-100 beats per minute. Subsequent to

the acclimatization period, recording was started in response to L-alanine. The amplitude of electrophysiological recordings from the olfactory epithelium is highly variable unless the detection procedure for the recording position and optimal distance between olfactory epithelium and recording electrode are standardized as much as possible. I therefore adhered to the following standardization procedure: Electrode position was adjusted three times within the area outlined in Figure 5.2 and the position with the highest response amplitude to a 10^{-3} M concentration of L-alanine was chosen. Once the "maximum response recording location" had been established, I determined the optimal distance between recording electrode and olfactory epithelium. To achieve response intensity curves that represent the EOG response amplitude to a range of odorant concentrations, test objects were stimulated with five concentrations of L-alanine, $MnCl_2$ (from $10^{-7.5}$ to 10^{-2} M) and cholic acid (from $10^{-9.4}$ to $10^{-4.2}$ M), respectively. The concentration range for $MnCl_2$ was chosen arbitrarily because no information could be found concerning electrophysiological responses to this odorant. The ranges for L-alanine and cholic acid were chosen to correspond with detection threshold limits reported by Hara et al. (1984). All odorants were delivered for ten seconds and the concentration profile of the odorants during valve opening was determined in dye tests with methylene blue (Figure 4.3.). To obtain a response intensity curve, the highest concentration of each odorant was presented first and decremented every two trials. To confirm the first two responses to each odorant, the intensity steps were repeated from the lowest to the highest concentration.

Statistical analysis

Within each treatment group, nine to ten fish were tested and the mean and the standard error of the response amplitude of the whole treatment group were determined at each concentration. The distribution of values around the mean for each concentration was in all cases tested for normality. To search for significant differences between response intensity curves obtained from different treatment groups the "repeated measures ANOVA" procedure (SPSS Base 10.0 Applications Guide, 1999) was used. The repeated measures ANOVA required that differences occur along the whole concentration range to be statistically significant. P-values $\leq$ 0.05 were taken to indicate a significant difference between response intensity curves of two treatment groups.

Treatment groups

The following treatment groups were chosen to elucidate the relationship between hormonal action, state of maturity and odorant exposure history (Table 5.1). To test for artifacts, I determined the EOG response of three dead fish to each of the three odorants (Group A). The other treatment groups could be divided into two categories. Category one consisted of fish that had never been exposed to artificially increased concentrations of the odorants that were used as stimuli in the EOG recordings and were defined as naïve fish. Category two consisted of fish that were exposed to artificially increased concentrations of $MnCl_2$ and a combined T_3T_4 treatment for 30 days, starting 90 days after first morphological signs of PST.

Table 5.1.: Treatment schedule and testing groups for the EOG recordings.

Age	Activity	Odorant and Hormone exposed fish	Control Fish
Month 0	Rearing	No exposure	No exposure
↓			
Month 11 (90 days after morphological signs of PST)	Exposure	$MnCl_2$ and T_3+T_4 exposure for 30 days	No exposure
↓			
Month 12 until Month 35	Rearing	No exposure during rearing	No exposure during rearing
Month 36-38: EOG response recorded to: **1. cholic acid 2. L-alanine 3. $MnCl_2$** in the following groups:	Testing	Group 1: immature Group 2: mature	Group A: control test for artifacts of odours on dead fish Group 3: immature Group 4: immature, tested after 30 days of T_3+T_4 treatment Group 5: immature, tested after two injections with GnRH Group 6: mature fish

V.3. Chapter 5: Results

Artifact control tests

A control experiment was carried out to ensure that none of the three odorants would interact with the type of electrode used, and thus produce artifacts that could not be distinguished from the real EOG responses. EOG responses to all three odorants were recorded twice from the same fish, first when the fish were alive and for the second time after the fish had been euthanized and frozen for three days. Any response recorded in dead fish was assumed to be an artifact rather than an electrophysiological response initiated by the depolarization of ORNs. L-alanine at a concentration of $10^{-3.4}$ M elicited a strong response amplitude of 3.9 mV in living fish (black trace, Figure 5.3.a) while no artifacts in response to L-alanine could be detected in dead fish (gray trace, Figure 5.3.a). The electrophysiological response to cholic acid was similar. A strong EOG response was recorded (amplitude 5 mV) in response to cholic acid ($10^{-4.2}$ M) in living fish (black trace, Figuer 5.3.b), while no response artifacts were visible in dead fish (gray trace, Figure 5.3.b). In contrast, when recording EOG responses to $MnCl_2$ in dead fish (gray trace, Figure 5.3.c), an artifact amplitude of 52% of the response in living fish (black trace, Figure 5.3.c) was recorded. The phenomenon was observed at this strength only at the highest concentration of $10^{-3.4}$ M. For concentrations lower than 10^{-4} M the artifact component of the whole response dropped to 30%. Since the artifact component at concentrations higher than 10^{-4} M made up more than half of the whole response, I did not consider EOG responses to this concentration for the response intensity curves.

Figure 5.3.a.

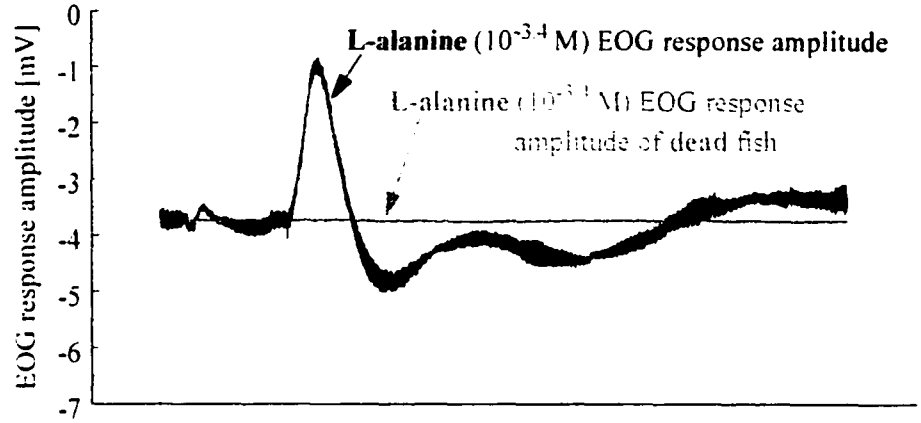


Figure 5.3.b.

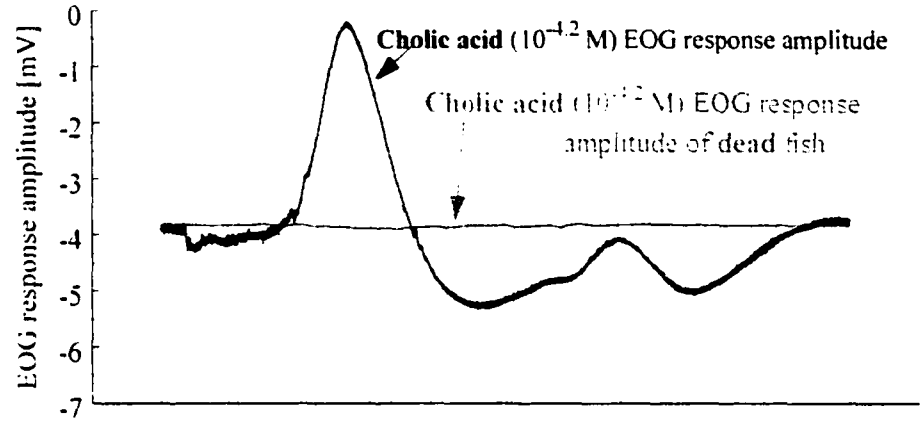


Figure 5.3.c.

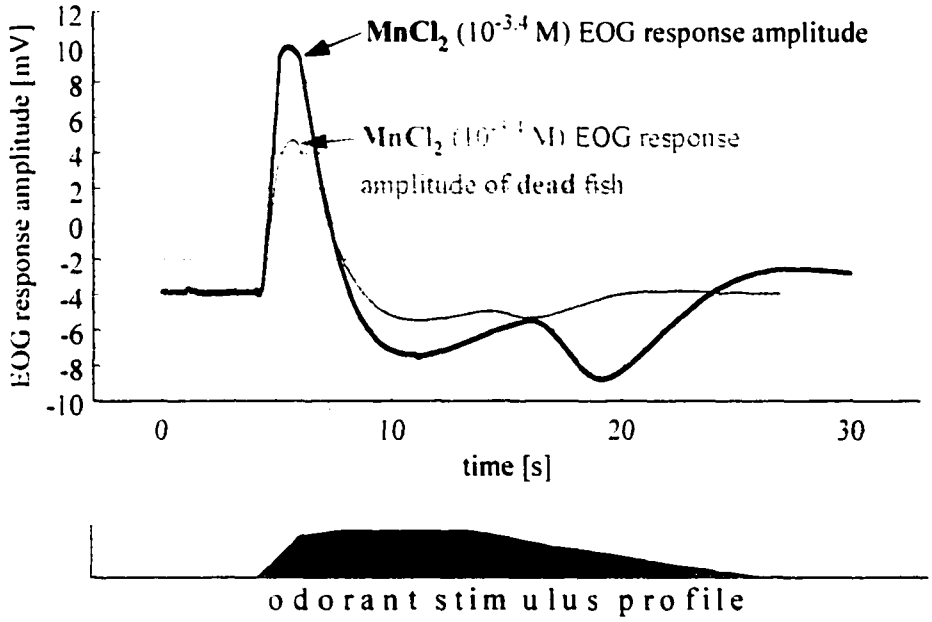


Figure 5.3. a., b., c.: (legend next page):

Figure 5.3. a., b. and c.: In all three figures EOG example traces in response to an odorant (y-axis) are plotted against time in seconds (x-axis). The black trace is recorded from a living fish while the gray trace is recorded from the same fish after killing and three days of freezing. Overall, I recorded from five fish, first alive and then dead. The traces shown are typical for each odorant tested and represent the results for all five fish that were tested per odorant.
Figure 5.3.a. clearly demonstrates that L-alanine at a concentration of $10^{-3.4}$ M elicits a strong response with an amplitude of 3.9 mV in living fish (black trace). Artifacts in response to L-alanine (gray trace) could not be detected in dead fish.
In Figure 5.3.b. the same expected result is shown. While a strong EOG response (amplitude 5 mV) was recorded to cholic acid ($10^{-4.2}$ M) in living fish (black trace), no response artifacts were visible in dead fish (gray trace).
Figure 5.3.c.: In response to $MnCl_2$ ($10^{-3.4}$ M), I recorded an artifact (gray trace) with an amplitude of 52% of the response in living fish (black trace). Below Figure 5.3.c., the odorant stimulus profile was plotted over time to show the fast onset and gradual diminishing of the olfactory stimulus.

For concentrations lower than 10^{-4} M the results were adjusted by 30% to obtain absolute amplitude values for response intensity curves. For L-alanine and cholic acid the values did not have to be adjusted and responses to the whole concentration range could be considered for the response intensity curve.

EOG responses from naïve immature versus naïve mature sockeye salmon

I recorded EOG responses to the odorants L-alanine, cholic acid and $MnCl_2$ in immature and mature naïve sockeye salmon at three years of age to test for possible modulations of the EOG response by the state of maturity.

No significant difference in the EOG response to L-alanine could be found when immature and mature fish were compared ($p = 0.126$, Figure 5.4.a) but the response amplitude in mature fish seemed higher than in immature fish at the two highest concentrations.

Figure 5.4.a.:

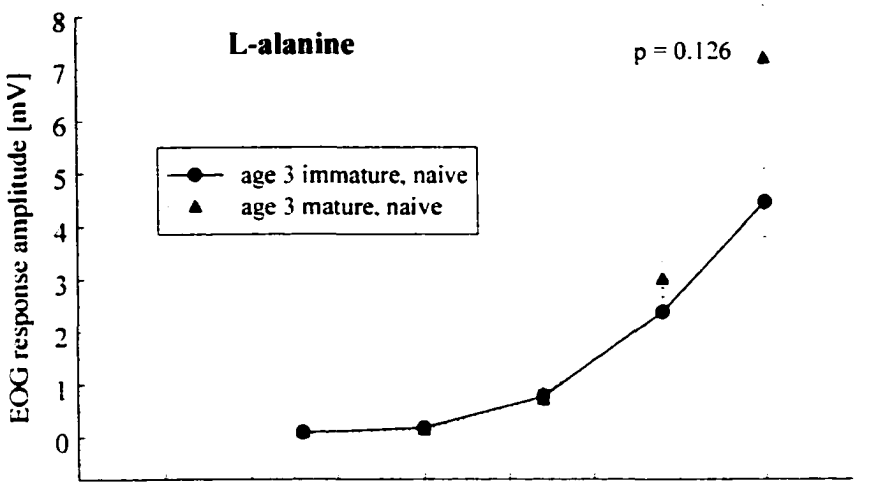


Figure 5.4.b.:

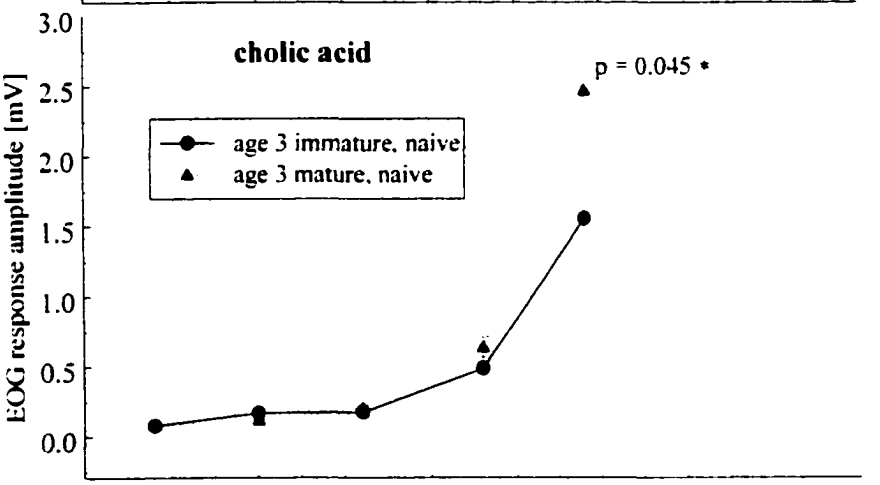


Figure 5.4.c.:

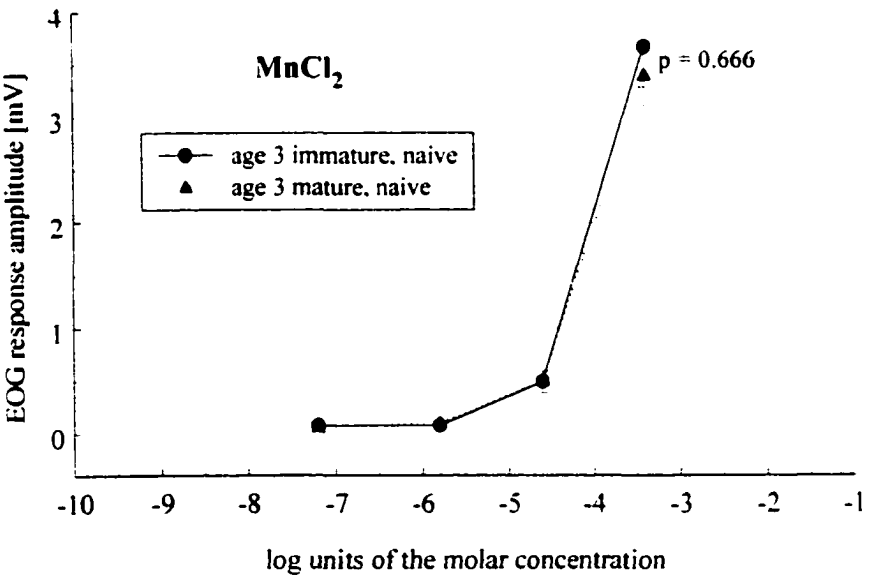


Figure 5.4.a, b and c (legend next page

Figure 5.4.a., b. and c.: In **Figure 5.4.a.** the EOG response amplitude to L-alanine is plotted against the odorant concentration ranging from 10^{-2} to $10^{-7.4}$ M in five steps. Two treatment groups were considered, three-year-old immature fish (N=7,solid line with filled black circles for mean values) and three-year-old mature fish (N=9, stippled line with black triangles for mean values). The symbols for treatment groups apply to all three graphs within Figure 5.4. Error bars stand for the standard error of the mean. No significant difference in the sensitivity to L-alanine between immature and mature sockeye salmon could be found ($p = 0.126$). The response amplitude to cholic acid, shown in **Figure 5.4.b.**, was recorded to a concentration range from $10^{-9.4}$ to $10^{-4.2}$ M. The sensitivity difference between immature and mature sockeye salmon to cholic acid was significant (repeated measures ANOVA, $p = 0.045$*). The EOG response amplitude to $MnCl_2$ (**Figure 5.4.c.**) over a range from $10^{-7.2}$ to $10^{-3.4}$ M did not change significantly when immature and mature fish at three years of age were compared (repeated measures ANOVA, $p = 0.666$).

Overall, this led to a response intensity curve with a higher slope. The response amplitude to cholic acid, shown in Figure 5.4.b. was recorded over a concentration range from $10^{-9.4}$ to $10^{-4.2}$ M because the overall sensitivity to bile acids is several orders of magnitude higher than to amino acids (Hara et al., 1984). EOG response amplitude to cholic acid differed significantly between immature and mature sockeye salmon (repeated measures ANOVA, $p = 0.045$*). Similar to L-alanine, the gradient of the response amplitude curve to cholic acid was considerably steeper in mature fish. The EOG response amplitude in response to $MnCl_2$, shown in Figure 5.4.c., did not differ significantly between immature and mature fish (repeated measures ANOVA, $p = 0.666$).

In summary, EOG response to the organic compound cholic acid was enhanced by maturation, whereas the sensitivity to the inorganic $MnCl_2$ was not.

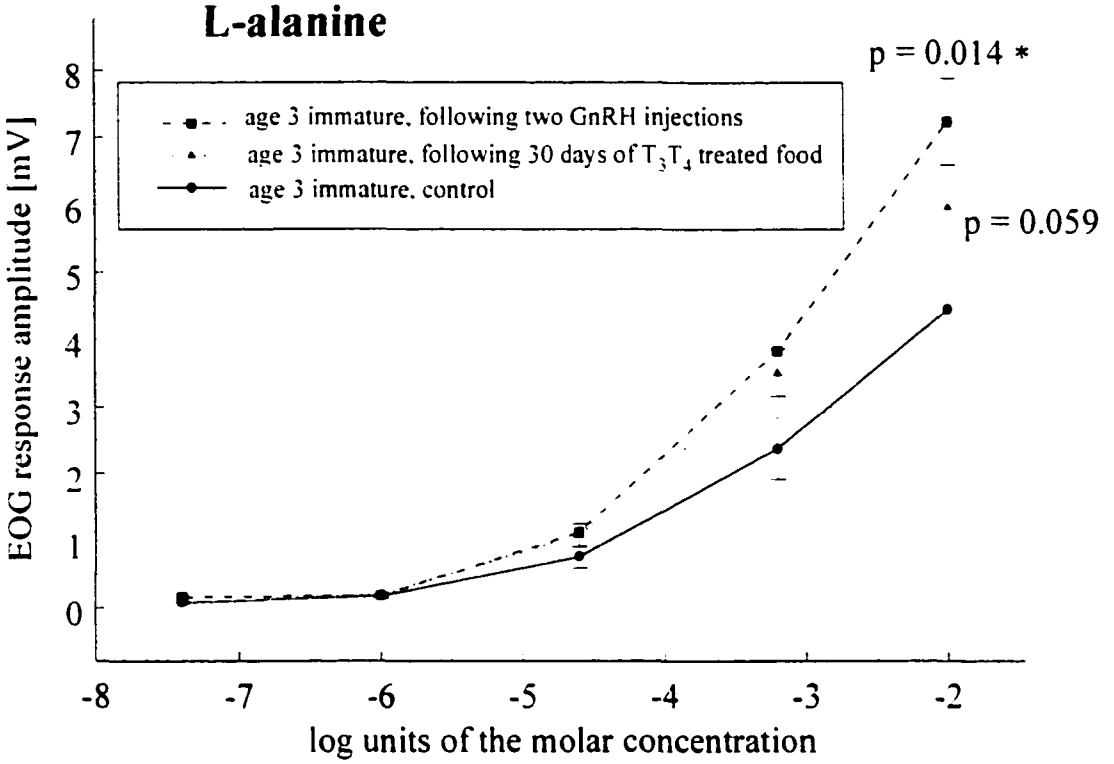


Figure 5.5.: Comparative EOG amplitude in response to a range of L-alanine concentrations in three year old immature control (circles connected by solid line), T_3T_4 treated (triangles connected by regularly stippled line) or GnRH treated (squares connected by irregularly stippled line) sockeye salmon. T_3T_4 treatment did not result in a significant change (repeated measures ANOVA, $p = 0.059$) of the olfactory sensitivity to L-alanine over the chosen concentration range (10^{-2} to $10^{-7.4}$ M). The response amplitude to L-alanine, subsequent to GnRH treatment, was increased over the whole range of concentrations which led to a significant ($p = 0.013$) difference between the treatment groups (error bars stand for the prevailing standard error of the mean).

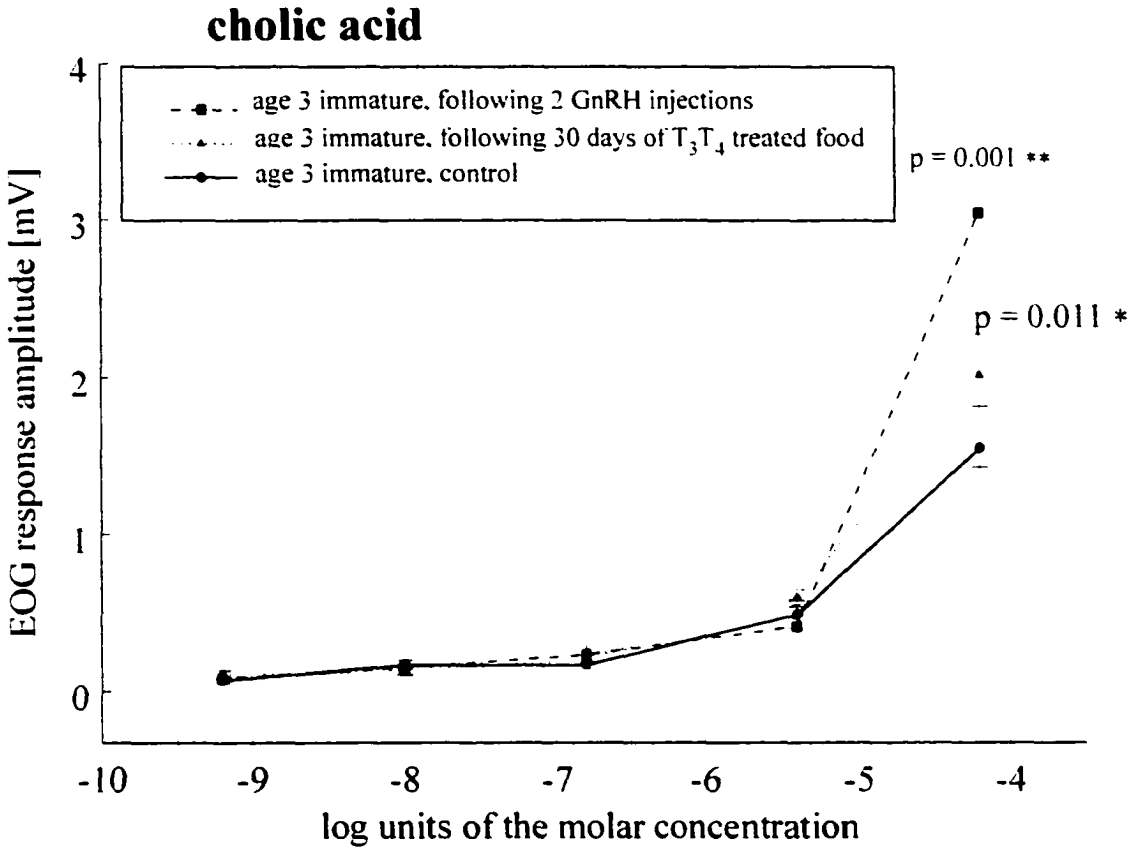


Figure 5.6.: Comparative EOG amplitude in response to a range of cholic acid concentrations in three year old immature control (circles connected by solid line). T_3T_4 treated (triangles connected by regularly stippled line) or GnRH treated (squares connected by irregularly stippled line) sockeye salmon. T_3T_4 treatment did result in a significant increase (repeated measures ANOVA, $p = 0.011$) of the EOG response amplitude to cholic acid over the chosen concentration range ($10^{-4.2}$ to $10^{-9.2}$ M). The response amplitude to cholic acid subsequent to GnRH treatment was also overall significantly increased ($p = 0.001$).

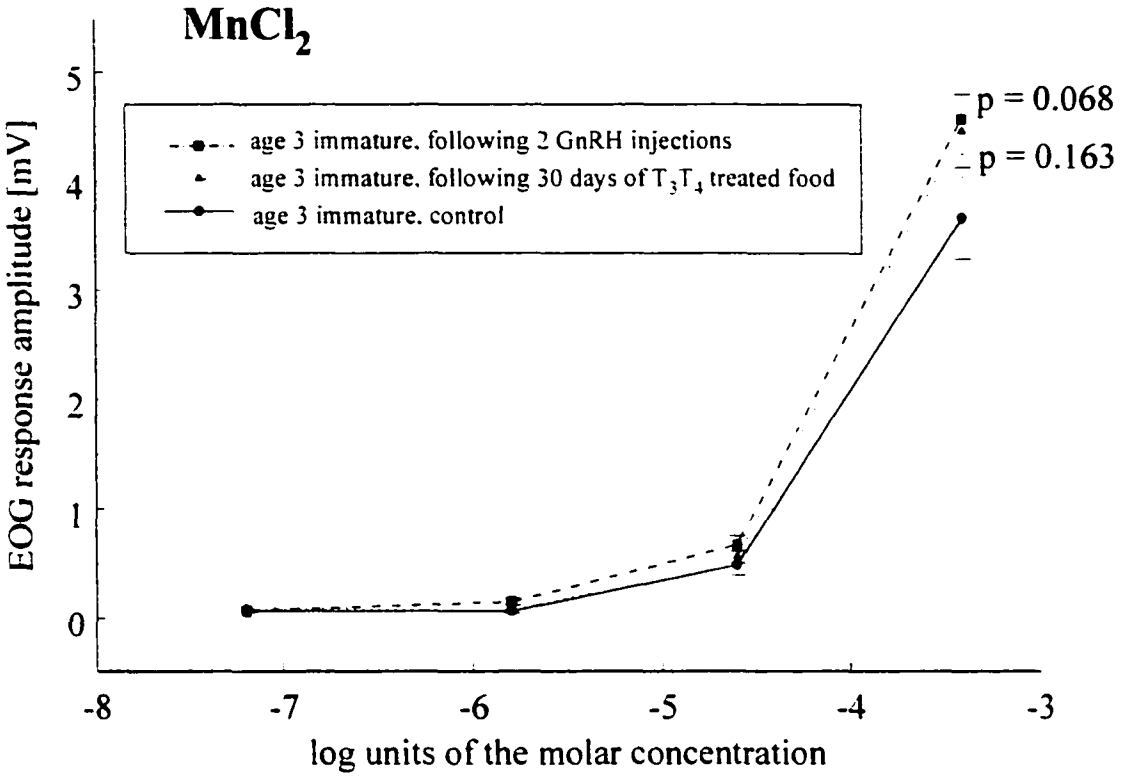


Figure 5.7.: Comparative EOG amplitude in response to a range of $MnCl_2$ concentrations in three year old immature control (circles connected by solid line), T_3T_4 treated (triangles connected by regularly stippled line) or GnRH treated (squares connected by irregularly stippled line) sockeye salmon. Neither T_3T_4, nor GnRH treatment resulted in significant changes (repeated measures ANOVA, $p = 0.163$ and $p = 0.068$ respectively) in olfactory sensitivity to $MnCl_2$ over the chosen concentration range ($10^{-3.4}$ to $10^{-7.2}$ M) when compared with values obtained from the control group (error bars stand for the prevailing standard error of the mean).

Is olfactory sensitivity in adult and immature sockeye salmon influenced by hormonal action of T_3T_4 or GnRH ?

Data presented in the previous section of this chapter suggests modulation of the EOG response by maturation. The next experimental step was to evaluate whether T_3T_4 and/or GnRH could also act as peripheral olfactory sensitivity modulators, given that both

have been suggested as mediators of olfactory modulation and are involved in many developmental processes (Cyr & Eales, 1996).

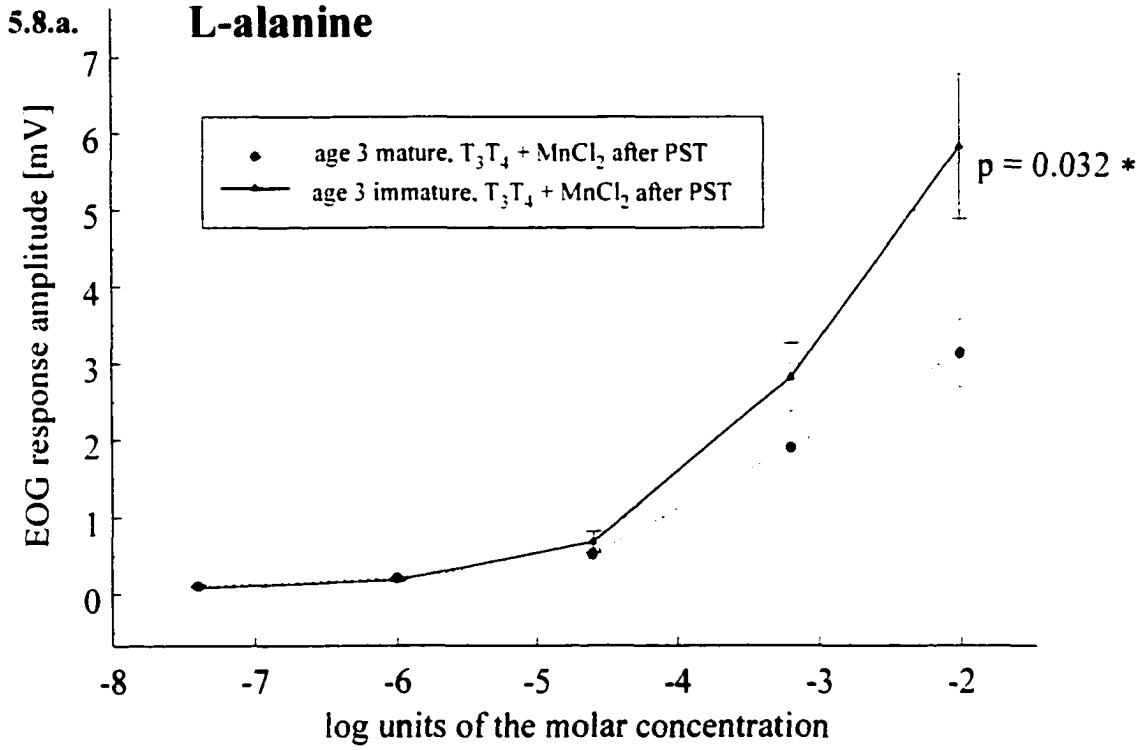
T_3T_4 treatment did not cause significant change (repeated measures ANOVA, $p = 0.059$) of the EOG response amplitude to L-alanine (Figure 5.5). Nevertheless, the steepness of the curve seemed increased at higher concentrations. The EOG amplitude in response to L-alanine after GnRH treatment increased significantly ($p = 0.013$), compared with control fish.

T_3T_4 and GnRH treatment increased the EOG response to cholic acid significantly ($p = 0.011$ and $p = 0.001$ respectively) (Figure 5.6.) but no hormonal sensitization was detected in the EOG responses to $MnCl_2$ (Figure 5.7). Neither T_3T_4 nor GnRH treatment resulted in significant changes (repeated measures ANOVA, $p = 0.163$ and $p = 0.068$ respectively) in the EOG response.

In summary, I conclude that changes in EOG responses caused by maturation or GnRH treatment are qualitatively and quantitatively similar. However, results from T_3T_4 treatment were equivocal and it would be premature to assume that thyroid hormones can induce peripheral olfactory modulation in three-year-old immature sockeye salmon.

Does juvenile treatment with thyroid hormones lead to olfactory imprinting?

In this section, I present the results of EOG recordings of fish that experienced exposure to $MnCl_2$ and T_3T_4 after the termination of the PST, two years before the recordings were conducted. Two treatment groups were tested for EOG responses to L-alanine, three-year-old immature fish (N=8) and three-year-old mature fish (N=9) (Figure 5.8.a).



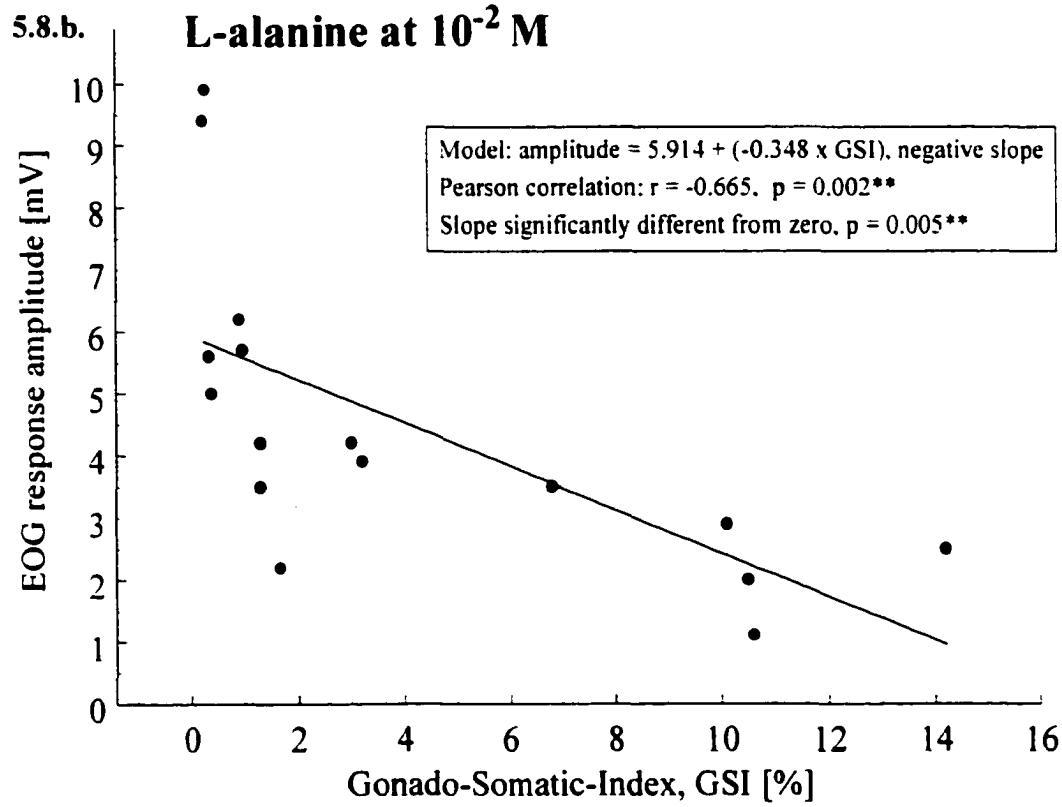


Figure 5.8.a. & b.: (legend next page)

Figure 5.8.a. & b: In Figure 5.8.a. the EOG response amplitude is plotted against L-alanine concentrations ranging from 10^{-2} to $10^{-7.4}$ M. Considered treatment groups are three-year-old immature fish (N=8,solid line with filled black circles for mean values) and three-year-old mature fish (N=9, stippled line with black triangles for mean values). Both groups of fish had been exposed to $MnCl_2$ and T_3T_4 for 30 days, starting 90 days after first morphological signs of PST. The EOG response amplitude in mature fish is significantly ($p = 0.032$) lower than in immature fish.
Figure 5.8.b. relates EOG amplitude (y-axis) at a 10^{-2} M concentration of L-alanine to the maturity state of the fish (x-axis) expressed in the gonado-somatic-index (GSI). The Pearson correlation between the two variables has a r-value of 0.665 and is highly significant ($p=0.002$). The slope of the linear relationship is strongly negative ($p = 0.005$).

The EOG response amplitude was significantly ($p = 0.032$) lower in mature fish than in immature fish and curve slope of mature fish appeared flatter. Moreover, EOG response to a 10^{-2} M concentration of L-alanine was significantly negatively correlated ($r = -0.665$, $p = 0.002$) with the gonado-somatic-index (GSI), the ratio of gonad weight to total body weight (Figure 5.8.b).

In contrast, the EOG response amplitude to cholic acid was significantly ($p = 0.033$) higher in mature than in immature fish (Figure 5.9.a). The EOG response amplitude to a fixed cholic acid concentration of $10^{-5.4}$ M was positively correlated ($r = 0.674$, $p = 0.003$) with the GSI.

Figure 5.9.a.

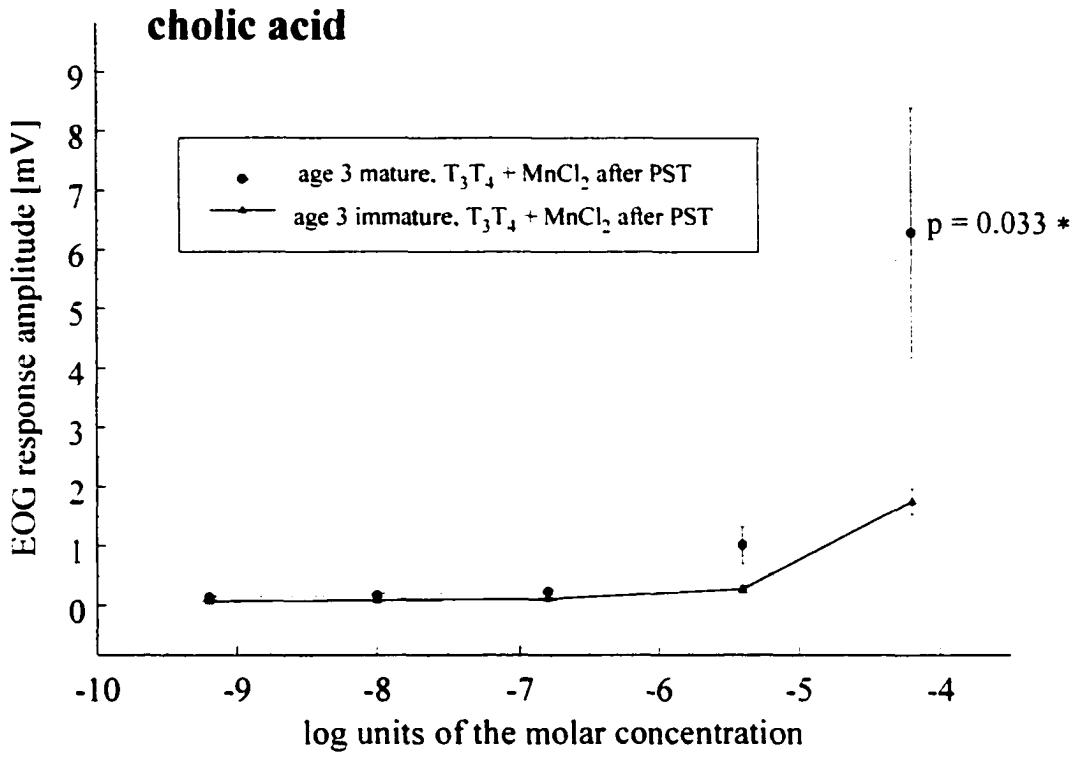


Figure 5.9.b.

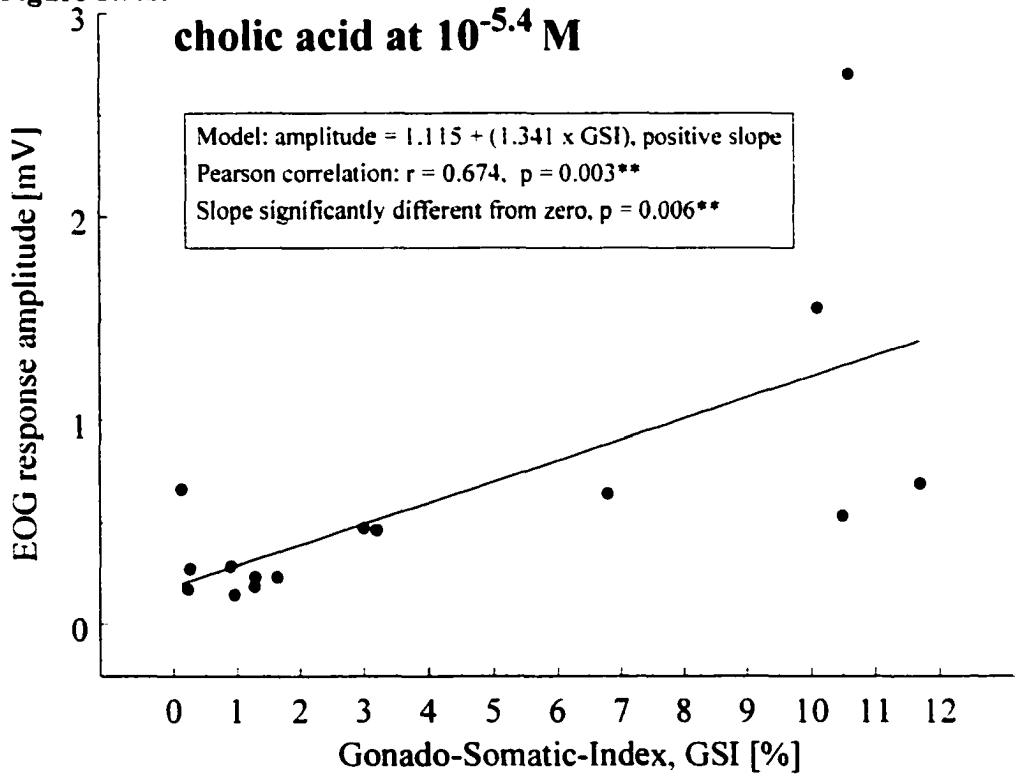


Figure 5.9.a. & b.: (legend next page)

Figure 5.9.a. & b: In Figure 5.9.a. the EOG response amplitude is plotted against cholic acid concentrations ranging from $10^{-4.2}$ to $10^{-9.2}$ M. Three-year-old immature fish (N=8, solid line with filled black circles for mean values) and three-year-old mature fish (N=9, stippled line with black triangles for mean values) are considered. Both groups of fish had been exposed to $MnCl_2$ and T_3T_4 for 30 days, starting 90 days after first morphological signs of PST. The EOG response amplitude in mature fish is significantly ($p = 0.033$) higher than in immature fish.
Figure 5.9.b. relates EOG amplitude (y-axis) in response to a $10^{-5.4}$ M concentration of cholic acid to maturity state (x-axis) expressed in the gonado-somatic-index (GSI) which describes the ratio of gonad weight to total body weight. The Pearson correlation between the two variables has a r-value of 0.674 and is highly significant ($p=0.003$). The slope of the linear relationship is strongly positive ($p = 0.006$).

EOG responses to $MnCl_2$ were significantly higher ($p = 0.0001$) in mature fish than in immature fish (Figure 5.10.a) and the gradient of the response amplitude curve appeared to be steeper for mature fish over the full concentration range. The EOG response to a fixed $10^{-3.4}$ M concentration of $MnCl_2$ was significantly positively correlated ($r = 0.588$, $p = 0.007$) with the GSI (Figure 5.10.b.)

In summary, for fish that had imprinted on $MnCl_2$, EOG response amplitudes increased significantly at maturity in response to both cholic acid and to the imprinting odorant $MnCl_2$, but decreased significantly at maturity in response to L-alanine.

Figure 5.10.a. $MnCl_2$

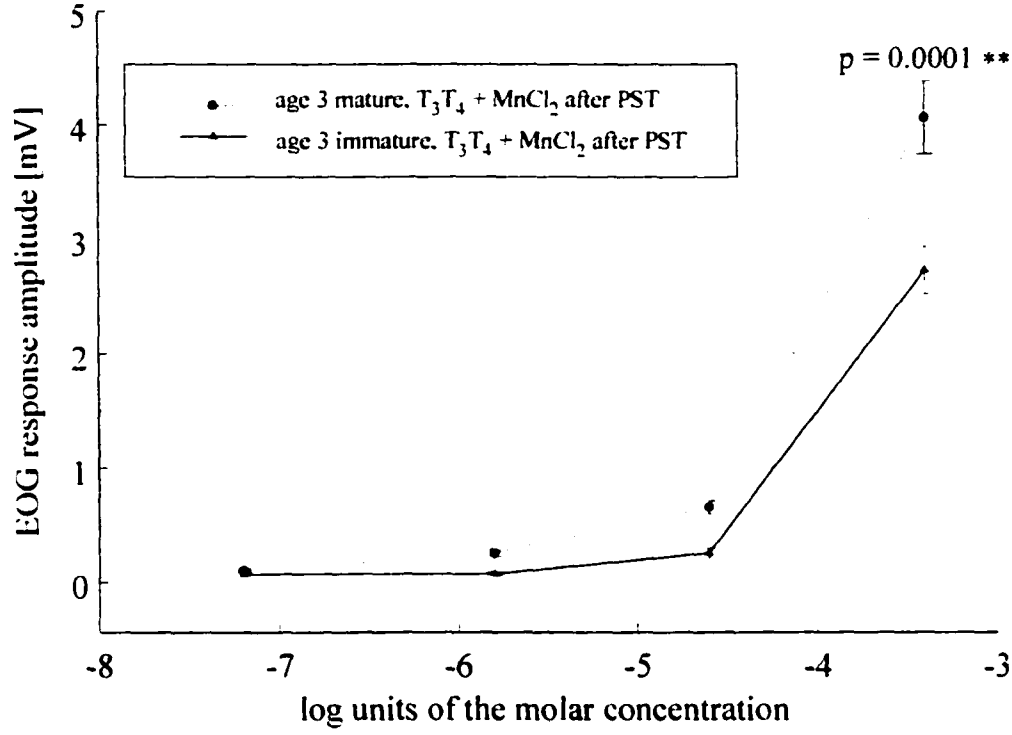


Figure 5.10.b. $MnCl_2$ at $10^{-3.4}$ M

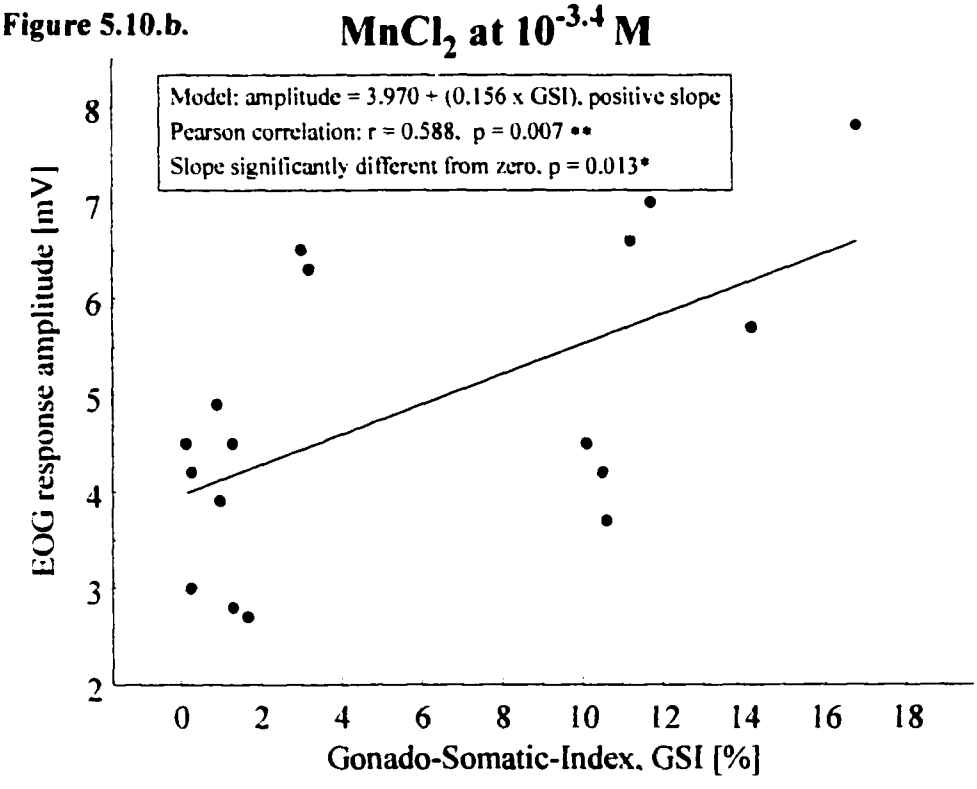


Figure 5.10. a. & b.: (legend next page)

Figure 5.10.a. & b: In Figure 5.10.a. the EOG response amplitude is plotted against $MnCl_2$ concentrations ranging from $10^{-3.4}$ to $10^{-7.2}$ M. Two treatment groups are considered. three-year-old immature fish (N=8,solid line with filled black circles for mean values) and three-year-old mature fish (N=9, stippled line with black triangles for mean values). Both groups of fish have been exposed to $MnCl_2$ and T_3T_4 for 30 days, starting 90 days after first morphological signs of PST. The EOG response amplitude in mature fish is significantly ($p = 0.0001$) higher than in immature fish.
Figure 5.10.b. relates the amplitude of the EOG response (y-axis) elicited by a constant concentration of $MnCl_2$ ($10^{-3.4}$ M) to the maturity state of the fish (x-axis) expressed in the gonado-somatic-index (GSI). The Pearson correlation between the two variables has a r-value of 0.588 and is highly significant ($p=0.007$). The slope of the linear relationship is significantly positive ($p = 0.013$).

Does imprinting modulate the EOG response amplitude?

To answer this question, EOG responses were recorded in three-year-old immature and mature sockeye salmon with two different histories of odorant exposure. Group one included naïve control fish that had never been exposed to artificially increased concentrations of any of the test odorants nor any kind of hormonal challenge. Group two included fish that had been exposed to T_3T_4 and $MnCl_2$ following the PST and were therefore called imprinted.

For immature fish, the EOG amplitude to $MnCl_2$ was significantly higher ($p = 0.027$) in the control group than in the imprinted group (Figure 5.11.a). In contrast, for mature fish, sensitivity to $MnCl_2$ was significantly higher ($p = 0.05$) in the imprinted group than in the control group (Figure 5.11.b.).

Figure 5.11.a.

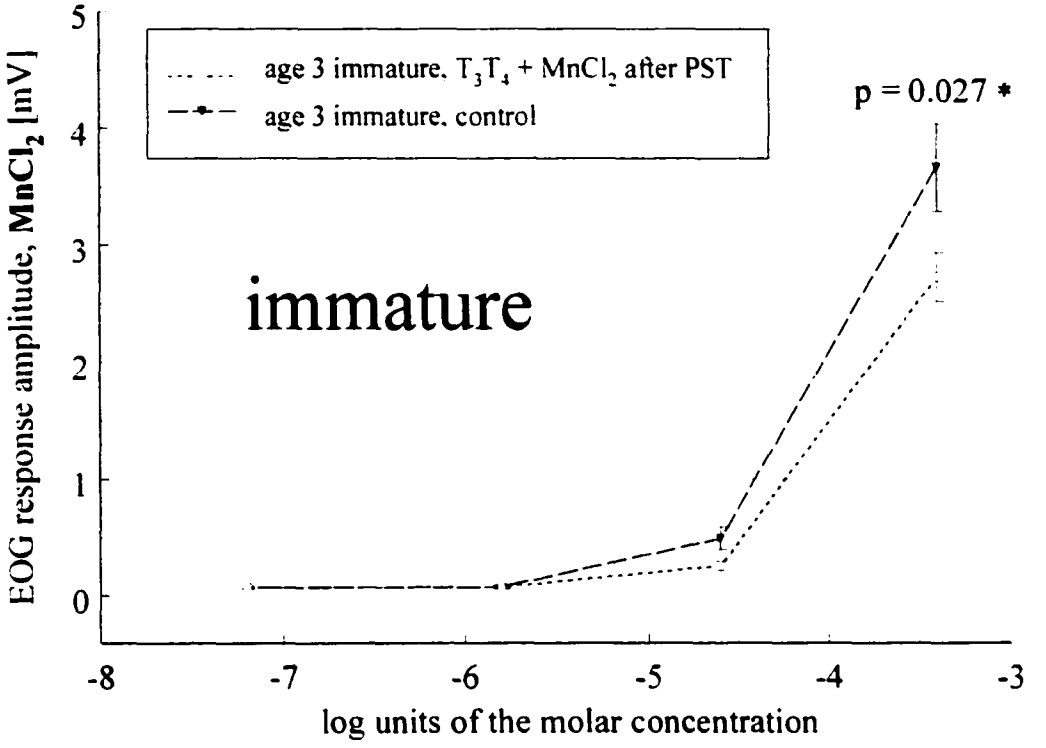


Figure 5.11.b.

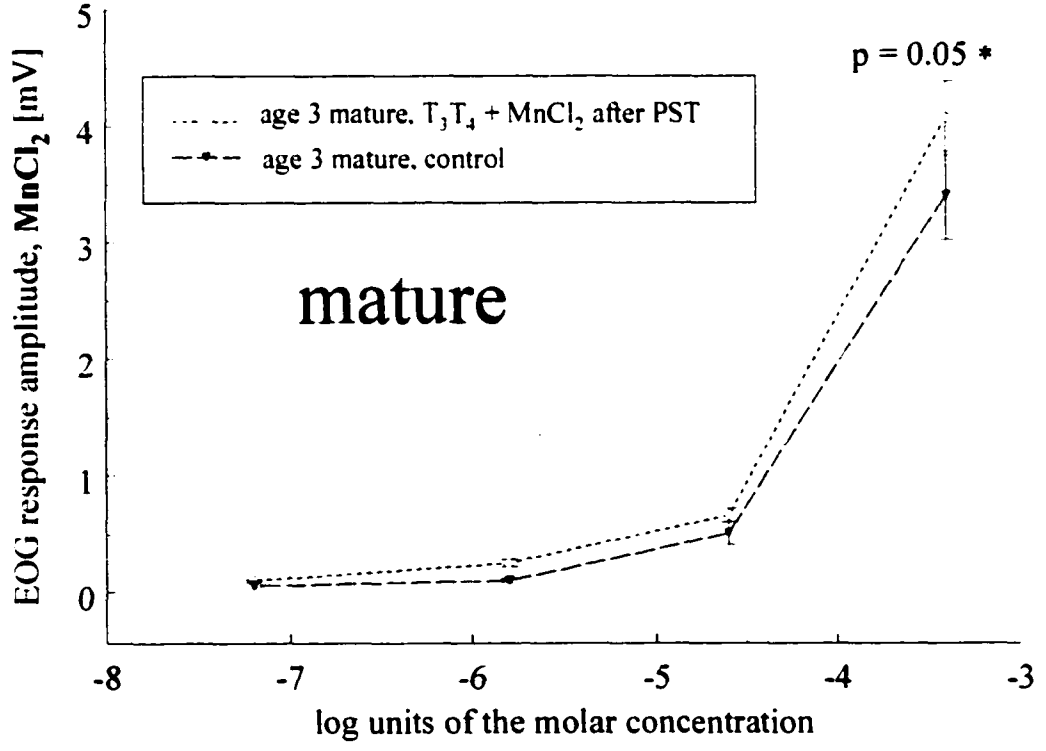


Figure 5.11.a. & b.: (legend next page)

Figure 5.11.a. & b: In both Figures 5.11.a. and 5.11.b. the EOG response amplitude in mV is plotted against a concentration range ($10^{-3.4}$ to $10^{-7.4}$M) of $MnCl_2$ in negative log units. In Figure 5.11.a. I compare immature naïve control fish (means are shown as upside down triangles connected by striped line) with immature and $MnCl_2$ imprinted fish (means are shown as open black circles connected by dotted line). Immature and imprinted sockeye salmon (Figure 5.11.a.) display a significantly ($p = 0.027$) reduced EOG response amplitude while the EOG response amplitude in mature and imprinted fish (Figure 5.11.b.) is significantly ($p = 0.05$) increased when compared to naïve control fish.

In the last experimental step, I examined whether the increase in EOG response amplitude in imprinted fish relative to that in control fish occurred over the whole concentration range or was particularly strong at the original imprinting concentration. The highest percentage increase in amplitude (257 %) difference between immature and mature imprinted fish occurred at a $MnCl_2$ concentration of 10^{-6} M, the concentration that the fish were exposed to during imprinting (Figure 5.12.a). To confirm whether the same phenomenon could be observed when mature naive fish were compared to mature imprinted fish, I compared EOG amplitude differences for each concentration between the two cohorts in Figure 5.12.b. Again, the highest percentage increase in amplitude (150%) occurred at a $MnCl_2$ concentration of 10^{-6} M, the imprinting concentration.

Figure 5.12.a.:

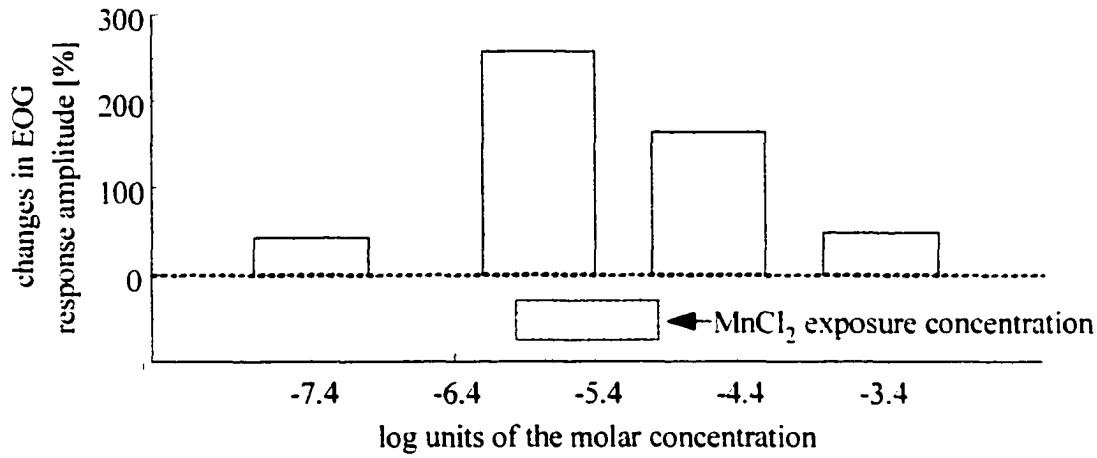


Figure 5.12.b.:

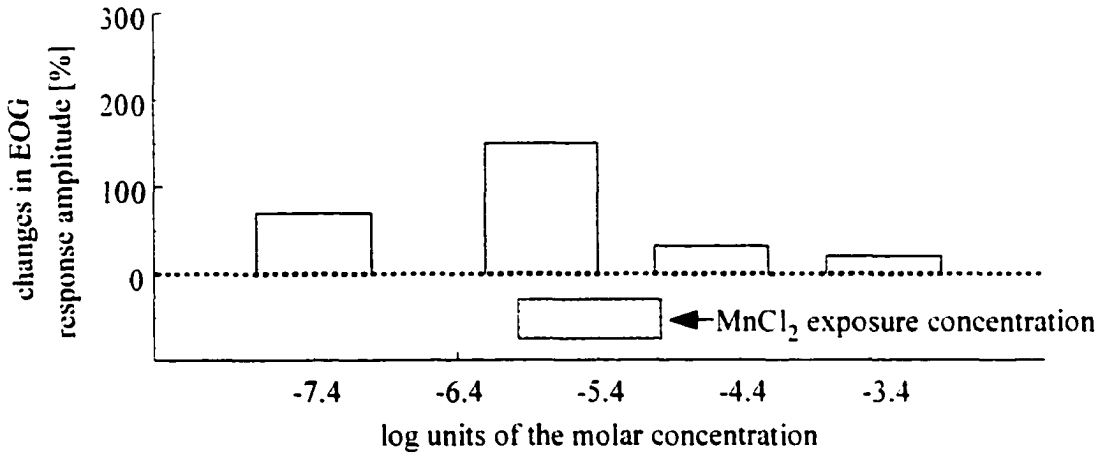


Figure 5.12.a, b: In Figure 5.12.a and b. I plotted changes in the EOG response amplitude (y-axis) for each of the test concentrations (x-axis). In Figure 5.12.a, EOG amplitudes are compared between immature imprinted and mature imprinted sockeye salmon (relating response amplitude curve shown in Figure 5.10.a). The strongest EOG amplitude difference (257 %) was observed in response to a $MnCl_2$ concentration around 10^{-6} M. The striped bar, below the result bars, stands for the $MnCl_2$ concentration during imprinting (Figures 5.12.a.and b)..
Figure 5.12.b. shows EOG amplitude differences between mature naïve and mature imprinted fish at different concentrations (relating response amplitude curve shown in Figure 5.11.b). The largest amplitude gain (150 %) occurred in response to a $MnCl_2$ concentration around 10^{-6} M.

V. 4. Chapter 5: Discussion

EOG recordings from sockeye salmon not previously exposed to test odorants:

The first experiments described in this chapter were designed to determine EOG responses of naïve fish to the three test odorants L-alanine, cholic acid and $MnCl_2$. Control responses were monitored to enable careful evaluation of the influence of state of maturity, GnRH and T_3 and T_4. The results were equivocal. EOG response amplitudes to the organic compound cholic acid changed significantly at maturity whereas the response amplitudes to L-alanine and $MnCl_2$ did not change significantly at maturity. Therefore, it seems that the state of maturity does not have the same effect on all odorant-binding receptor populations. My results also revealed that maturity does not cause a uniform increase in EOG response amplitude over the whole concentration range. Instead, it selectively enhances the response amplitude to higher odorant concentrations leading to an overall increase in the slope of response intensity curves. The steeper slope of a response amplitude curve may indicate improved resolution of odorant concentrations within the concentration range of ORNs. The consistency of the phenomenon for treatment groups in which olfactory sensitivity changed significantly, supports this assumption. The suggestion of plasticity in ORN function at maturity is in accordance with recent findings by Eisthen et al. (2000) who discovered neuromodulation of ORNs by GnRH, and with the fact that production and circulation of GnRH increases at maturity (Dickhoff & Swanson, 1990; Cyr & Eales, 1996). GnRH can increase inward currents into ORNs, thereby lowering the resting potential and thus possibly enhancing their excitability (Eisthen et al., 2000). Therefore, I hypothesize that the modulated sensitivity in mature fish was based on the hormonal action of GnRH. My hypothesis is

consistent with the observation that EOG responses were similar in GnRH treated and mature fish. Treatment with GnRH significantly increased EOG responses to L-alanine and cholic acid but not responses to $MnCl_2$.

Having found evidence for the neuromodulatory role of GnRH in ORNs of naïve sockeye salmon, I proceeded to record EOGs following treatment with T_3 and T_4. No significant change in the EOG responses to L-alanine and $MnCl_2$ could be detected, but the enhanced response amplitude to cholic acid suggests that T_3T_4 treatment can modify EOG response to an organic compound. However, T_3T_4 treatment was less potent than GnRH in this respect. The fact that peripheral olfactory sensitivity to $MnCl_2$ was not affected by maturity, GnRH or T_3T_4 treatment was an important finding for the experiments, in which $MnCl_2$ was used as an imprinting odorant.

In summary, GnRH and maturity affected peripheral olfactory sensitivity to organic compounds but not to $MnCl_2$.

EOG recordings from sockeye salmon previously exposed to test odorants:

In the next part of the study I tested for EOG responses in fish known to have imprinted to $MnCl_2$ under the influence of T_3T_4 as demonstrated in behavioural tests (Chapter 2). Although these fish had imprinted to $MnCl_2$ (no previous exposure to L-alanine and cholic acid) EOG responses were recorded to all three test odorants. Proceeding this way, I could monitor changes in sensitivity to non-imprinting odorants as well as the imprinting odorant. In fish imprinted to $MnCl_2$, the EOG response to L-alanine was significantly reduced in mature fish while the response to cholic acid was significantly increased at maturity, when compared with immature fish from the same

cohort. The maturity-induced increase in sensitivity to cholic acid in imprinted fish corroborates my earlier findings that maturity and GnRH increase the EOG response towards cholic acid in naïve fish. In contrast, the decreased response amplitude to L-alanine at maturity is not consistent with the results for naïve fish. One may be able to explain this phenomenon by considering the natural use of the odorant and the hormonal circumstances of the imprinting process. L-alanine is an amino acid found in most processed fish food and many natural food items (Sutterlin, 1975; Jones, 1989). Amongst the spectrum of amino acids that elicit one or more components of food search behaviour, L-alanine attracts the widest range of fish species including herring (*Clupea harengus*, Dempsey, 1978), Atlantic salmon (*Salmo salar*, Mearns, 1985) and brown trout (*Salmo trutta*) (Mearns, 1986). L-alanine can therefore be related to food searching behaviour, which ceases when Pacific salmon enter their home stream. When I artificially increased T_3T_4 levels to initiate the juvenile imprinting process, the high T_3T_4 levels might have imprinted $MnCl_2$ as one of the predominant components of the tank water. At the same time, imprinting may have caused the sensitivity to background odorants like L-alanine, to drop at maturity. This might result in an increased ability to distinguish the predominant imprinting odorant. If this hypothesis is correct, the sensitivity to $MnCl_2$, the imprinting odorant, should increase in a manner that sets it apart from maturity-induced changes in olfactory sensitivity to the other two odorants. The increase in sensitivity to $MnCl_2$ in mature imprinted sockeye compared to immature imprinted fish was highly significant ($p = 0.0001$). The fact that no hormonal or maturity-induced sensitization to $MnCl_2$ could be observed in naïve fish supports the assumption that the process of imprinting, rather than a general maturity-induced sensitization, was responsible for this

highly significant sensitization to the imprinting odorant $MnCl_2$. Moreover, the highest percentage increase in EOG amplitude occurred at a $MnCl_2$ concentration of 10^{-6} M which resembles the imprinting concentration. Thus, I have found evidence in EOG recordings supporting Nevitt's and Dittman's (1998) theory of peripheral sensitization to imprinting odorants in general and to the imprinting odorant concentration in particular. In addition to confirming Nevitt's and Dittman's results, by a different recording technique, this study has demonstrated that peripheral sensitization is related to T_3T_4 treatment during the imprinting period.

In the last experiment, EOG response amplitudes towards the imprinting odorant $MnCl_2$ were compared between naïve and imprinted sockeye salmon. When I analyzed data from immature fish, it emerged that the EOG response was significantly weaker in imprinted, than in non-imprinted test fish. This result supports one of my earlier behavioural results (Chapter II), in which immature imprinted fish showed less motivation to overcome a waterfall treated with the imprinting odorant than naïve fish. Thus, behavioural patterns and olfactory perception in imprinted fish change at maturity and differ from those in naïve fish. In nature, many migratory stocks of salmonids feed or reside close to the mouth of their natal stream (i.e., kokanee in Kootenay Lake) but do not enter the stream until they approach maturity. From an evolutionary perspective, it might be disadvantageous to enter the natal stream prematurely since conceivably only during the main run time could the genetic pool be preserved without mixing and competition for suitable spawning habitat. Motivational state (Chapter II) and olfactory perception (Chapter V), which appear to be under hormonal control and dependent on proper imprinting may be the mechanisms controlling migration timing. The comparison of

EOGs of imprinted and non-imprinted sockeye salmon at maturity support this theory. Imprinted sockeye salmon were significantly more sensitive to $MnCl_2$ than naïve fish and the strongest EOG amplitude gain was again observed in response to the imprinting concentration. Although the concept of sensitization to a particular imprinting odorant concentration is supported by my results, it can also be explained alternatively. The greatest potential for change in the response to an odorant is expected to occur in the dynamic range of its particular receptor population. In my experiments, the imprinting odorant concentration fell into the dynamic range of its receptors. Therefore, the apparent concentration specific sensitization may be caused by receptor sensitization to the whole concentration range.

In the remainder of this chapter, I suggest a model of imprinting that represents a synthesis of results from this and other studies. The cellular mechanism that leads to an imprinting-based sensitization of ORNs is likely triggered by the hormonal action of a combination of T_3 and T_4 (this study). The two forms of thyroid hormones may sensitize ORNs by binding to their nuclear receptors and initiating a process (Eales, 1995) that modulates guanylyl cyclase activity (Dittman et al., 1997). Thus, ORNs are sensitized to the predominant olfactory stimuli experienced during increased T_3 and T_4 availability for ORNs. The odorant composition, specific to the natal stream, must then be internalized to be recovered several years later to guide homing in fresh water. Since thyroid hormones decline throughout maturation in nature (Cyr & Eales, 1996), another trigger must initiate ORN sensitization that motivates salmon to recognize and swim towards their natal stream shortly before maturation. For the following reasons this trigger may be GnRH: GnRH can enhance the enzymatic activity of deiodinases, which have the potential to

increase the availability of the physiologically active T_3 in the olfactory epithelium (this study, Chapter III). GnRH has neuromodulatory (this study) and motivational capabilities (Sato et al., 1997, this study Chapter II) that can promote the sensitization and behavioural response towards an imprinting odorant (this study). During homing in fresh water, the sensitivity to the imprinting is increased but in addition, the sensitivity to food-specific odorants might be reduced at the same time (this study).

Few details are known about an essential part of the olfactory imprinting and odorant recognition process, the long-term retention of imprinting related olfactory information. Nor is it known how imprinting related sensitization can be maintained in the peripheral olfactory system while ORNs are replaced continually (Farbman, 1994). Efferent fibers from the olfactory bulb or higher centers where the information could be stored, would allow for centrifugal feedback to the olfactory epithelium, but this mechanism has not yet been described. A likely candidate system for centrifugal feedback is the network of GnRH fibers that connect parts of the CNS directly with the olfactory epithelium in salmon (Parhar et al., 1994; Nevitt at al., 1995). This network is established early on in ontogeny in sockeye salmon (Parhar et al., 1995) but its function remains unclear. It has been suggested that they facilitate imprinting (Parhar et al., 1994) based on their distribution throughout the olfactory system and their increased expression at times of juvenile migration, which co-incides with T_3 and T_4 surges. Nevertheless, attempts to record electrophysiological responses of GnRH neurons to olfactory stimuli have been unsuccessful (Hara, personal communication). Given that GnRH neuron expression can coincide with high T_3 and T_4 levels (Parhar et al., 1994) and that GnRH increases deiodinase activity in sensory tissues (this study, Chapter III), GnRH neurons

might have a dual function in the context of imprinting. They might regulate T_3 availability in the olfactory epithelium and manifest the internalization of olfactory stimuli that are necessary for homing in a higher center of the olfactory system.

Since many questions about olfactory imprinting remain to be answered, I can only make suggestions that are securely based on experimental evidence but lack definite detail. I hope these suggestions and models will renew interest in the fascinating field of olfactory imprinting in migratory salmonids.

Chapter VI: Synthesis and future outlook

In synthesis, the combined studies of this thesis add to the existing knowledge about olfactory imprinting and imprinting based homing in the following way.
Both forms of thyroid hormones (T_3 and T_4) previously suggested to be important for olfactory sensitization leading to olfactory imprinting, have been shown to be essential. Only a combined T_3T_4 treatment reliably led to successful imprinting to an odorant in my studies and elevated the concentration of both hormones to levels that are experienced at the PST in nature (Chapter 2 & 3). Separate T_4 treatment did not raise the serum concentration of the physiologically active T_3 and did not trigger the imprinting process (Chapter 2 & 3). The developmental stage chosen for artificial imprinting appeared to be of no importance as long as T_3 and T_4 plasma levels were raised artificially to sufficient levels (Chapter 2 & 4). I therefore suggest that imprinting in nature occurs at the PST because of the increased levels of thyroid hormones at this developmental stage. Imprinting not only causes attraction to the imprinting odorant, it can also cause avoidance of the imprinting odorant when fish are not in the adequate hormonal state for homing (Chapter 2). The physiological state that triggers attraction to the imprinting odorant in adult fish can be achieved through GnRH treatment and occurs naturally at maturity (Chapter 2,4 & 5). Besides the motivational effect of GnRH for imprinting-odorant recognition and homing behaviour, GnRH also enhances the conversion of the physiologically inactive T_4 into the physiologically T_3. This conversion process has been demonstrated for the first time in the retina and the olfactory epithelium in this study (Chapter 3). Moreover, GnRH can lower the threshold concentration for the response to

an imprinting odorant in fish that have been imprinted under the influence of T_3 and T_4 (Chapter 4). The juvenile imprinting procedure, relying on a combined T_3T_4 treatment also caused far-reaching modulation of the peripheral sensitivity to imprinting and non-imprinting odorants as demonstrated in EOG recordings. EOG recordings proved useful to reveal a close link between physiological state of the fish and their sensitivity to an imprinting odorant (Chapter 5). Sensitivity to the imprinting odorant decreased in immature fish and increased in mature fish compared with the sensitivity in non-imprinted control fish (Chapter 5). Olfactory sensitivity to an odorant like L-alanine, related to feeding behaviour, which ceases during homing or mating, can be reduced by imprinting (Chapter 5). This might result in an increased ability to distinguish predominant olfactory stimuli used to guide homing from background odorants. Sensitivity to imprinting and non-imprinting odorants was likely modulated by hormonal action, since evidence for hormonal sensitization, triggered by GnRH and T_3T_4 was observed in adult fish regardless of their odorant exposure history (Chapter 5). My results, also revealed that maturity might increase the slope of EOG response intensity curves. An increased slope may enhance the ability to detect concentration changes within the dynamic range of ORNs (Chapter 5).

Based on this synthesis, I can suggest experimental approaches to address the most significant gaps in our understanding of olfactory imprinting in salmon.

It may be useful to employ molecular techniques to investigate whether thyroid hormones and/or GnRH initiate differential expression of enzymes in ORNs or even lead to enhanced ORN turnover. The possibility of hormone-based plasticity in ORN function is consistent with recent findings by Eisthen et al. (2000) who discovered a

neuromodulatory role of GnRH in ORNs of the mudpuppy (*Necturus maculosus*). Either by labeling GnRH or by local application of GnRH binding blockers it should be possible to clarify the role and mode of action of GnRH. Then it would be possible to decide whether the retention of olfactory information can be accomplished on the level of the ORNs or whether interaction between the olfactory epithelium and higher centers within the olfactory system is necessary. To investigate whether the action of GnRH is restricted to the olfactory systems or whether overall muscle activity is altered as well, I suggest that motor neuron activity in response to an imprinted odorant should be compared following saline or GnRH treatment.

Aside from hormonal affects, it is still unknown which group of odorants is used for olfactory imprinting in nature. A behavioural arena, in which naturally homing salmon could respond in an easily quantifiable way to water from their home stream, could be used as a control experiment. Changes in this behavioural response, brought about by elimination or substitution of certain components of the home stream water, could possibly demonstrate the importance of certain odorant groups for home stream recognition.

On the electrophysiological level, voltage sensitive dyes would be an effective tool to determine whether imprinting can modulate the spatial pattern of depolarization in the olfactory epithelium and/or the olfactory bulb. This way specificity of ORNs to certain odorants could be mapped on two levels of computation and changes related to imprinting or hormonal treatments could possibly be elucidated.

And to go back to the roots of homing research, telemetry is needed to observe behavioural patterns during the approach to the spawning stream in nature. I think it is

unlikely that salmon orient towards a concentration gradient created by water from a typically small natal stream during homing in very large rivers like the Fraser or the Skeena River. Moreover, constant exposure to the imprinting odorant experienced while homing should lead to adaptation of the ORNs binding the imprinting odorant or odorants. In contrast, I believe that under those conditions changes in concentration of the imprinting scent of several orders of magnitude would be easier to detect. In other words, I hypothesize that fish overshoot their natal stream until the natal stream odorant drops from a high concentration to non-detectable level, then retrace their path to make the correct choice. This behavioural pattern should be visible using telemetry with a high temporal and spatial resolution. As a last recommendation, I think that the classic imprinting experiments conducted by Hasler and colleagues in the freshwater environment of the Great Lakes should be repeated in sea-going salmonid stocks. So far, all attempts to repeat these experiments in anadromous fish with an oceanic growth phase have been unsuccessful.

Bibliography

Alexander, G., Sweeting, R. and McKeown, B. (1994). The shift in visual pigment dominance in the retinae of juvenile coho salmon (*Oncorhynchus kisutch*): an indicator of smolt status. *Journal of Experimental Biology* **195**, 185-197.

Arnold, A. P. (1992). Hormonally-induced alterations in synaptic organization in adult nervous system. *Experimental Gerontology* **27**, 99-110.

Barinaga, M. (1999). Salmon follow watery odors. *Science* **286**, 705-706.

Beauchamp, R. D. and Rowe, J. S. (1977). Goldfish spectral sensitivity: A conditioned heart-rate measure in restrained or curarized fish. *Vision Research* **17**, 617-624.

Biddiscombe, S. (1983). Plasma levels of thyroid hormones in sockeye salmon (*Oncorhynchus nerka*) decrease before spawning. *General and Comparative Endocrinology* **52**, 467-470.

Blackbourn, D. J. (1987). Sea surface temperature and pre-season prediction of return timing in Fraser River sockeye salmon (*Oncorhynchus nerka*). *Canadian Special Publications of Fisheries and Aquatic Sciences* **96**, 296-306.

Bodznick, D. (1978 a). Characterization of olfactory bulb units of sockeye salmon with behaviourally relevant stimuli. *Journal of Comparative Physiology* **127**, 147-155.

Bodznick, D. (1978 b). Calcium ion: an odorant for natural water discriminations and the migratory behaviour of sockeye salmon. *Journal of Comparative Physiology* **127**, 139-146.

Breer, H., Klemm, T. and Boekhoff, I. (1992). Nitric oxide mediated formation of cyclic GMP in the olfactory system. *Neuroreport* **3**, 1030-1032.

Browman, H. I. and Hawrhyshyn, C. W. (1992). Throxine induces a precocial loss of ultraviolet photesensitivity in rainbow trout (*Oncorhynchus mykiss*, Teleostei). *Vision Research* **32**, 2303-2312.

Buck, L. and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odour recognition. *Cell* **65**, 175-187.

Burgner, R. L. (1991). Life history of sockeye salmon (*Oncorhynchus nerka*). In *Pacific salmon life histories* (ed. C. Groot and L. Margolis), pp. 2-117. Vancouver: UBC Press.

Burton, S., Murphy, D., Uzma, Q., Sutton, P. and O'Keefe, J. (2000). Combined lesions of hippocampus and subiculum do not produce deficits in a nonspatial social olfactory memory task. *The Journal of Neuroscience* **20**, 5468-5475.

Cahill, L. and McGaugh, J. L. (1998). Mechanisms of emotional arousal and lasting declarative memory. *Trends in Neuroscience* **21**, 294-299.

Chang, J. P., Yu, K. L., Wong, A. O. L. and Peter, R. E. (1990). Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. *Neuroendocrinology* **51**, 664-674.

Chrisp, E. Y. and Bjorn, T. C. (1978). Parr-smolt transformation and seaward migration of wild and hatchery steelhead trout in Idaho. *Federal Aid to Fish and Wildlife Restoration Project, Project F-49-12, Salmon and Steelhead Investigations* .

Clasen, J. (1998). Efficiency control of particle removal by rapid sand filters in treatment plants fed with reservoir water: a survey of different methods. *Water Science Technology* **37**, 19-26.

Cooper, J. C., Hara, T. J. and Brown, S. B. (1982). Letters and Comments: Comment on electroencephalographic responses to morpholine and their relationship to homing. *Canadian Journal Of Fisheries and Aquatic Sciences* **39**, 1544-1548.

Cooper, J. C. and Hasler, A. D. (1973). An electrophysiological approach to salmon homing. Nanaimo, British Columbia: Pacific Biological Station.

Cooper, J. C. and Hasler, A. D. (1974). Electrophysiological evidence for retention of olfactory cues in homing coho salmon. *Science* **183**, 336-338.

Cooper, J. C. and Hasler, A. D. (1975). Morpholine as olfactory stimulus in fish. *Science* **187**, 81-82.

Cooper, J. C. and Hasler, A. D. (1976). Electrophysiological studies of morpholine-imprinted coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **33**, 688-694.

Cooper, J. C. and Hirsch, P. J. (1982). The role of chemoreception in salmonid homing. In *Chemoreception in fishes* (ed. T. J. Hara), pp. 343-362. Amsterdam: Elsevier Science Publication Company.

Cooper, J. C. and Scholz, A. T. (1976). Homing of artificially imprinted steelhead trout. *Journal of the Fisheries Research Board of Canada* **33**, 826-829.

Cyr, D. G. and Eales, J. G. (1996). Interrelationships between thyroidal and reproductive endocrine systems in fish. *Reviews in Fish Biology and Fisheries* **6**, 165-200.

Dempsey, C. H. (1978). Chemical stimuli as a factor in feeding and intraspecific behaviour of herring larvae. *Journal of marine biological Assessment U.K.* **58**, 739-747.

Denver, R. J. (1998). The molecular basis of thyroid hormone-dependent central nervous system remodeling during amphibian metamorphosis. *Comparative Biochemistry and Physiology Part C* **119**, 219-228.

Dickhoff, W. W. and Sullivan, C. (1987). Involvement of the thyroid gland in smoltification, with special reference to metabolic and developmental processes. *American Fisheries Society Symposium* **1**, 197-210.

Dickhoff, W. W. and Swanson, P. (1990). Functions of salmon pituitary glycoprotein hormones: "The maturational surge hypothesis". In *Progress in Comparative Endocrinology* (ed. A. Epple, C. G. Scanes and M. H. Stetson), pp. 349-356. New York: Wiley-Liss, Incorporated.

Dingle, H. (1996). Migration. The biology of life on the move, pp. 474. New York, Oxford: Oxford University Press.

Dittman, A. H. and Quinn, T. P. (1996). Homing in Pacific salmon: mechanisms and ecological basis. *Journal of Experimental Biology* **199**, 83-91.

Dittman, A. H., Quinn, T. P., Dickhoff, W. W. and Larsen, D. A. (1994). Interactions between novel water, thyroxine and olfactory imprinting in underyearling coho salmon (*Oncorhynchus kisutch* Walbaum). *Aquaculture and Fisheries Management* **25 (Supplement 2)**, 157-169.

Dittman, A. H., Quinn, T. P. and Nevitt, G. A. (1996). Timing of impriting to natural and artificial odors by coho salmon, *Onchorhynchus kisutch*. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 434-442.

Dittman, A. H., Quinn, T. P., Nevitt, G. A., Hacker, B. and Storm, D. R. (1997). Sensitization of olfactory guanylyl cyclase to a specific odorant in coho salmon. *Neuron* **19**, 381-389.

Donaldson, E. M., Hunter, G. A. and Dye, H. M. (1981). Induced ovulation in coho salmon (*Oncorhynchus kisutch*). *Aquaculture* **26**, 129-141.

Doving, K. B., Westerberg, H. and Johnsen, P. B. (1985). Role of olfaction in the behavioral and neuronal responses of Atlantic salmon, *Salmo salar*, to hydrographic stratification. *Canadian Journal of Fisheries and Aquatic Sciences* **42**, 1658-1667.

Eales, J. G. (1979). Thyroid function in cyclostomes and fishes. In *Hormones and Evolution*, vol. 1 (ed. E. J. Barrington), pp. 341-436. New York: Academic Press.

Eales, J. G. (1995). Regulation and measurement of thyroidal status in fish. *Netherlands Journal of Zoology* **45**, 175-180.

Eales, J. G. and Brown, S. B. (1993). Measurement and regulation of thyroidal status in teleost fish. *Reviews in Fish Biology and Fisheries* **3**, 299-347.

Eales, J. G., Brown, S. B., Cyr, D. G., Adams, B. A. and Finnson, R. (1999). Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish. In *Environmental Toxicology and Risk Assessment*, vol. 8 (ed. D. S. Henshel, M. C. Black and M. C. Harass), pp. 136-164. West Conshohocken, PA, USA: American Society for Testing and Materials.

Eisthen, H. L., Delay, R. J., Wirsig-Wiechmann, C. R. and Dionne, V. E. (2000). Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. *The Journal of Neuroscience* **20**, 3947-3955.

Farbman, A. I. (1994). Developmental biology of olfactory sensory neurons. *Seminars in Cell Biology* **5**, 3-10.

Foerster, R. E. (1968). The sockeye salmon, *Oncorhynchus nerka*. *Bulletin of the Fisheries Research Board of Canada* **162**, 422.

Grau, E. G., Dickhoff, W. W., Nishioka, R. S., Bern, H. A. and Folmar, L. C. (1981). Lunar phasing of the thyroxine surge preparatory to seaward migration of salmonid fish. *Science* **211**, 607-609.

Groot, C. (1972). Migration of yearling sockeye salmon (Oncorhynchus nerka) as detrmined by time-lapse photography of sonar observation. *Journal of the Fisheries Rsearch Board of Canada* **29**, 1431-1444.

Groot, C. and Margolis, L. (1991). Pacific Salmon Life Histories, pp. 564. Vancouver: UBC Press.

Hara, T. J. (1967b). Electrophysiological studies of the olfactory system of the goldfish, Carrassius auratus L.. Effects of sex hormones on the electrical activity of the olfactory bulb. *Comparative Biochemistry and Physiology* **22**, 209-226.

Hara, T. J. (1970). An electrophysiological basis for olfactory discrimination in homing salmon: a review. *Journal of the Fisheries Research Board of Canada* **27**, 565-586.

Hara, T. J. (1974). Is morpholine an effective olfactory stimulant in fish? *Journal of the Fisheries Research Board of Canada* **31**, 1547-1550.

Hara, T. J. (1976). Effects of pH on the olfactory responses to amino acids in rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* **54 A**, 37-39.

Hara, T. J. and Brown, S. B. (1979). Olfactory bulbar electrical response of rainbow trout (*Salmo gairdneri*) exposed to morpholine during smoltification. *Journal of the Fisheries Research Board of Canada* **36**, 1186-1190.

Hara, T. J., Macdonald, S., Evans, R. E., Marui, T. and Arai, S. (1984). Morpholine, bile acids and skin mucus as possible chemical cues in salmonid homing: electrophysiological re-evaluation. In *Mechanisms of Migration in Fishes* (ed. J. D. McCleave, G. P. Arnold, J. D. Dodson and W. H. Neill): Plenum Publishing Corporation.

Hara, T. J. and Zhang, C. (1998). Topographic bulbar projections and dual neural pathways of the primary olfactory neurons in salmonid fishes. *Neuroscience* **82**, 301-313.

Harden-Jones, F. R. (1968). Fish migration, pp. 325. London: Arnold Press.

Hasler, A. D. (1966). Underwater guideposts: homing of salmon, pp. 155. Madison: University of Wisconsin Press.

Hasler, A. D. and Scholz, A. T. (1980). Artificial imprinting: a procedure for conserving salmon stocks. In *Fish behavior and its use in the capture and culture of fishes*, vol. 5 (ed. J. Bardach, J. J. Magnuson, R. C. May and J. M. Reinhart), pp. 179-199. Manila: ICLARM Conference Proceedings, Center for Living Aquatic Resources Mangement, Manila, Philipines.

Hasler, A. D. and Scholz, A. T. (1983). Olfactory imprinting and homing in salmon, pp. 133. Berlin, Heidelberg, New York: Springer Verlag.

Hasler, A. D., Scholz, A. T. and Horrall, R. M. (1978). Olfactory imprinting and homing in salmon. *American Scientist* **66**, 347-355.

Hasler, A. D. and Wisby, W. J. (1951). Discrimination of stream odors by fishes and relation to parent stream behavior. *American Naturalist* **85**, 223-238.

Hawryshyn, C. W. (1991). Spatial summation of cone mechanisms in goldfish. *Vision Research* **31**, 563-566.

Hawryshyn, C. W. (1992). Polarization vision in fish. *American Scientist* **80**, 164-175.

Hawryshyn, C. W. and Beauchamp, R. (1985). Ultraviolet photosensitivity in goldfish: An independent U.V. retinal mechanism. *Vision Research* **25**, 11-20.

Hazon, N. and Balment, R. J. (1998). Endocrinology. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 441-463. Boca Raton, Florida: CRC Press.

Hirsch, P. J. (1977). Conditioning the heart rate of coho salmon (*Oncorhynchus kisutch*) to odors, pp. 89. Madison: University of Wisconsin.

Hoar, W. S. (1976). Smolt transformation: evolution , behaviour, and physiology. *Journal of the Fisheries Research Board of Canada* **33**, 1233-1252.

Hoar, W. S. (1988). The physiology of smolting salmonids. In *Gills, anatomy, gas transfer and acid-base regulation*, vol. 11B (ed. W. S. H. a. D. J. Randall). New York: Academic Press.

Incorporated, S. (1999). SPSS Base 10.0 Applications Guide. Chicago, Ilinois: SPSS Incorporated.

Jones, K. A. (1989). The palatability of amino acids and related compounds to rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **34**, 149-160.

Jones, K. A. and Hara, T. J. (1985). Behavioural responses of fishes to chemical cues: results from a new bioassay. *Journal of Fish Biology* **27**, 495-504.

Joy, K. P., Senthilkumaran, B. and Sudhakumari, C. C. (1998). Periovulatory changes in hypothalamic and pituitary monoamines following GnRH analogue treatment in the catfish *Heteropneustes fossilis*: a study correlating changes in plasma hormone profiles. *Journal of Endocrinology* **156**, 365-372.

Madison, D. M., Scholz, A. T., Cooper, J. C. and Hasler, A. D. (1973). Olfactory hypothesis and salmon migration: a synopsis of recent findings. *Fisheries Research Board of Canada Technical Report* **414**, 37.

Mazeaud, F. (1981/1982). Morpholine, a nonspecific attractant for salmonids. *Aquaculture* **26**, 189-191.

McBride, J. R. (1967). Effects of feeding on the thyroid, kidney, and pancreas in sexually ripening adult sockeye salmon (*Oncorhynchus nerka*). *Journal of the Fisheries Research Board of Canada* **24**, 67-76.

Mearns, K. J. (1985). Response of Atlantic salmon (Salmo salar) yearlings to individual L-amino acids. *Aquaculture* **48**, 253-259.

Mearns, K. J. (1986). Sensitivity of brown trout (*Salmo trutta* L.) and Atlantic salmon (*Salmon salar* L.) fry to amino acids at the start of exogenous feeding. *Aquaculture* **55**, 191-200.

Morin, P.-P., Dodson, J. J. and Dore, F. Y. (1987). Heart-rate-conditioning to L-cysteine and other chemical stimuli in young Atlantic salmon (*Salmo salar*). *Canadian Journal of Zoology* **65**, 2710-2714.

Morin, P.-P., Dodson, J. J. and Dore, F. Y. (1989a). Cardiac response to a natural odorant as evidence of a sensitive period of olfactory imprinting in young Atlantic salmon, *Salmo salar*. *Canadian Journal of Fisheries and Aquatic Sciences* **46**, 122-130.

Morin, P.-P., Dodson, J. J. and Dore, F. Y. (1989b). Thyroid activity concomitant with olfactory learning and heart rate changes in Atlantic salmon, *Salmo salar*, during smoltification. *Canadian Journal of Fisheries and Aquatic Sciences* **46**, 131-136.

Morin, P.-P., Hara, T. J. and Eales, J. G. (1993). Thyroid hormone deiodination in brain, liver,gill,heart and muscle of Atlantic salmon, *Salmo salar*, during photoperiodically induced parr-smolt transformation. *General and Comparative Endocrininology* **90**, 142-156.

Morin, P.-P., Hara, T. J. and Eales, J. G. (1995). T_4 depresses olfactory responses to L-alanine and plasma T_3 and T_3 production in smoltifying Atlantic salmon. *American Journal of Physiology* **269**, R1434-1440.

Morin, P.-P., Hara, T. J. and Eales, J. G. (1997). Thyroid function and olfactory responses to L-alanine during induced smoltification in Atlantic salmon, *Salmo salar*. *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 596-602.

Mulligan, T. J., Lapi, L., Kieser, R., Yamada, S. B. and Duewer, D. L. (1983). Salmon stock identification based on elemental composition of vertebrae. *Canadian Journal of Fisheries and Aquatic Sciences* **40**, 215-229.

Nakamura, T. and Gold, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325**, 442-444.

Nevitt, G. and Dittman, A. (1998). A new model for olfactory imprinting in salmon. *Integrative Biology* **1**, 215-223.

Nevitt, G. A., Dittman, A. H., Quinn, T. P. and Moody, W. J. (1994). Evidence for peripheral olfactory memory in imprinted salmon. *Proceedings of the National Academy of Science of the USA* **91**, 4288-4292.

Nevitt, G. A., Grober, M. S., Marchaterre, M. A. and Bass, A. H. (1995). GnRH-like immunoreactivity in the peripheral olfactory system and forebrain of Atlantic salmon (*Salmo salar*): A reassessment at multiple life history stages. *Brain Behaviour and Evolution* **45**, 350-358.

Nevitt, G. A. and Moody, W. J. (1992). An electrophysiological characterization of ciliated olfactory receptor cells of the coho salmon, *Oncorhynchus kisutch*. *Journal of Experimental Biology* **166**, 1-17.

Nordeng, H. (1971). Is the local orientation of anadromous fish determined by pheromones? *Nature* **233**, 411-413.

Okubo, K., Suetake, H., Usami, T. and Aida, K. (2000). Molecular cloning and tissue-specific expresssion of a gonadotropin-releasing hormone receptor in the Japanese eel. *General and Comparative Endocrinology* **119**, 181-192.

Orozco, A., Linser, P. and Valverde, C. (2000). Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish, *Fundulus heteroclitus*. *Comparative Biochemistry and Physiology B* **126**, 283-290.

Ottoson, D. (1971). The electro-olfactogram. In *Chemical Senses Part 1*, vol. IV (ed. L. M. Beidler), pp. 95-131. Berlin: Springer Verlag.

Overmier, J. B. and Hollis, K. L. (1983). The teleostean telencephalon in learning. In *Fish Neurobiology*, vol. 2 (ed. R. E. Davis and R. G. Northcutt), pp. 265-284. Ann Arbor: The University of Michigan Press.

Parhar, I. S., Iwata, M., Pfaff, D. W. and Schwanzel-Fukuda, M. (1995). Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon. *The Journal of Comparative Neurology* **362**, 256-270.

Parhar, I. S., Koibuchi, N., Sakai, M., Iwata, M. and Yamaoka, S. (1994). Gonadotropin-releasing hormone (GnRH): expression during salmon migration. *Neuroscience Letters* **172**, 15-18.

Paulsen, O. and Moser, E. I. (1998). A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. *Trends in Neuroscience* **21**, 273-278.

Quinn, T. P. (1980). Evidence for celestial and magnetic compass orientation in lake migrating sockeye salmon fry. *Journal of Comparative Physiology* **137**, 243-248.

Quinn, T. P. (1982). A model for salmon navigation on the high seas. In *Salmon and trout migratory behavior symposium*. (ed. E. L. Brannon and E. O. Salo), pp. 309. Seattle, Washington, School of Fisheries, University of Washington: University of Washington.

Quinn, T. P. (1993). A review of homing and straying of wild and hatchery-produced salmon. *Fisheries Research* **18**, 29-44.

Quinn, T. P. and TerHart, B. A. (1987). Movements of adult sockeye salmon (*Oncorhynchus nerka*) in British Columbia coastal waters in relation to temperature and salinity stratification: ultrasonic telemetry results. *Canadian Special Publication of Fisheries and Aquatic Sciences* **96**, 61-77.

Quinn, T. P., Wood, C. C., Margolis, L., Riddell, B. E. and Hyatt, K. D. (1987). Homing in wild sockeye salmon (*Oncorhynchus nerka*) populations as inferred from differences in parasite prevalence and allozyme allele frequency. *Canadian Journal of Fisheries and Aquatic Sciences* **44**, 1963-1971.

Rami, A., Patel, A. J. and Rabie, A. (1986b). Thyroid hormone and development of the rat hippocampus: morphological alterations in granule and pyramidal cells. *Neuroscience* **19**, 1217-1226.

Rami, A., Rabie, A. and Patel, A. J. (1986a). Thyroid hormone and development of the rat hippocampus: cell acquisition in the dentate gyrus. *Neuroscience* **19**, 1207-1216.

Rehnberg, B. G., Curtis, L. and Schreck, C. B. (1985). Homing of coho salmon (*Oncorhynchus kisutch*) exposed to morpholine. *Aquaculture* **44**, 253-255.

Reisenbichler, R. R. (1988). Relation between distance transferred from natal stream and relative return rate for hatchery coho salmon. *North American Journal of Fisheries Management* **8**, 172-174.

Ricker, W. E. (1972). Hereditary and environmental factors affecting certain salmonid populations. In *The stock concept in Pacific salmon. H.R. MacMillan Lectures in Fisheries, University of British Columbia, Vancouver* (ed. R. C. Simon and P. A. Larkin). Vancouver: University of British Columbia.

Sato, A., Ueda, H., Fukaya, M., Kaeriyama, M., Zohar, Y., Urano, A. and Yamauchi, K. (1997). Sexual diffrences in homing profiles and shortening of homing duration by gonadotropin-releasing-hormone analog implantation in lacustrine sockeye salmon (*Oncorhynchus nerka*) in Lake Shikotsu. *Zoological Science* **14**, 1009-1014.

Scheer, B. T. (1939). Homing instinct in salmon. *Quarterly Review of Biology* **14**, 408-420.

Scholz, A. T. (1980). Hormonal regulation of smolt transformation and olfactory imprinting in coho salmon, pp. 363. Madison: University of Wisconsin.

Scholz, A. T., Gosse, C. K., Cooper, J. C., Horrall, R. M., Hasler, A. D., Daly, R. T. and Poll, R. J. (1978a). Homing of rainbow trout transplanted in Lake Michigan: A comparison of three procedures used for imprinting and stocking. *Transactions of the American Fisheries Society* **107**, 439-443.

Scholz, A. T., Horall, R. M., Cooper, J. C. and Hasler, A. D. (1976). Imprinting to chemical cues. The basis for homestream selection in salmon. *Science* **196**, 1247-1249.

Stabell, O. B. (1984). Homing and olfaction in salmonids: a critical review with special reference to the Atlantic salmon. *Biological Review* **59**, 333-388.

Stabell, O. B. (1992). Olfactory control of homing behaviour in salmonids. In *Fish Chemoreception* (ed. T. J. Hara), pp. 249-270. London: Chapman & Hall.

Sutterlin, A. M. (1975). Chemical attraction of some marine fish in their natural habitat. *Journal of the Fisheries Research Board of Canada* **32**, 729-738.

Suzuki, K., Kawauchi, H. and Nagahama, Y. (1988c). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *General and Comparative Endocrinology* **71**, 292-301.

Tallman, R. F., and M. C. Healey. 1994. Homing, straying, and gene flow among seasonally separated populations of chum salmon (*Oncorhynchus keta*). *Canadian Journal of Fisheries and Aquatic Sciences.* **51,** 577-588.

Taylor, E. B. (1991). A review of local adaptation in Salmonidae, with particular reference to Pacific and Atlantic salmon. *Aquaculture* **98,** 185-207.

Tilson, M. B., Scholz, A. T., White, R. J. and Galloway, H. (1994). Thyroid-induced chemical imprinting in early life stages and assessment of smoltification in kokanee salmon hatcheries. *1993 Annual Report, prepared for Bonneville Power Administration, Portland Oregon* .

Tilson, M. B., Scholz, A. T., White, R. J. and Henderickson, J. L. (1995). Artificial imprinting and smolting in juvenile kokanee salmon: implications for operating Lake Roosevelt kokanee salmon hatcheries. *1994 Annual Report, Prepared for Bonneville Power Administration, Portland, Oregon* .

Vacher, C., Mananos, E. L., Breton, B., Marmingnon, M. H. and Saligaut, C. (2000). Modulation of pituitary dopamine D1 or D2 receptors and secretion of follicle stimulating hormone and luteinizing hormone during annual reproductive cycle of female rainbow trout. *Journal of Neurendocrinology* **12,** 1219-1226.

Wallenstein, G. V., Eichenbaum, H. and Hasselmo, M. E. (1998). The hippocampus as an associator of discontiguous events. *Trends in Neuroscience* **21,** 317-323.

Westerberg, H. (1984). The orientation of fish and the vertical stratification at fine- and micro- structure scales. In *Mechanisms of fish migration* (ed. J. D. McCleave and e. al.), pp. 574. New York: Plenum Press.

Wiltschko, W., Wiltschko, R. and Munro U. (2000). Light dependent magnetoreception in birds: the effect of intensity of 565-nm green light. *Naturwissenschaften,* **87** (8), 366-369.

Wisby, W. J. and Hasler, A. D. (1954). The effect of olfactory occlusion on migrating silver salmon (*O. kisutch*). *Journal of the Fisheries Research Board of Canada* **11,** 472-478.

Wood, C. C. and Foote, C. J. (1996). Evidence for sympatric genetic divergence of anadromous and non-anadromous morphs of sockeye salmon (*Oncorhynchus nerka*). *Evolution* **50,** 1265-1279.

Wulliman, M. F. (1998). The Central Nervous System. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 247-282. Boca Raton, Florida: CRC Press.

Youngson, A. F. and Simpson, T. H. (1984). Changes in serum thyroxine levels during smolting in captive and wild Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology* **24**, 29-39.