Bile Acids as Potential Pheromones in Lake Char *Salvelinus namaycush*: An Electrophysiological, Biochemical and Behavioural Study

BY

CHUNBO ZHANG

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Department of Zoology
University of Manitoba
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BILE ACIDS AS POTENTIAL PHEROMONES IN LAKE CHAR *Salvelinus namaycush*: AN ELECTROPHYSIOLOGICAL, BIOCHEMICAL AND BEHAVIOURAL STUDY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Abstract

Bile acids are potent fish chemostimulants. They may play a role in mediating fish behaviour. Using electrophysiological, biochemical and behavioural approaches, I examined the hypothesis that bile acids function as pheromones in lake char *Salvelinus namaycush*. Electroolfactogram (EOG) recordings showed that 9 out of 38 authentic bile acids tested had detection thresholds of 0.02-0.5 nM. The most stimulatory bile acids included chenodeoxycholic acid (CD), cholic acid (CA), taurochenodeoxycholic acid (TCD), taurocholic acid (TCA) and taurolithocholic acid 3α-sulphate (TLS). Analysis of structure-activity relationships showed that olfactory sensitivity and specificity for bile acids were affected by 1) position and orientation of hydroxyls; 2) hydroxy sulphation; 3) side chain length and 4) side chain substituents of these molecules. Cross-adaptation and binary mixture experiments demonstrated that olfactory responses to bile acids were mediated by at least three types of receptors that were specific for free, amidated and sulphated bile acids. The olfactory receptors for bile acids were distinct from those for other known fish odorants. Using rosette tissue sections, [^{3}H]TCA autoradiographic binding showed the presence of three binding components that met receptor criteria. Olfactory neuron denervation and deciliation indicated that binding activity was directly associated with olfactory sensory neurons, especially the sensory cilia. Behaviourally, lake char preferred water containing bile at 1 nl/l, or TCA at 10^{-8}-10^{-7} M in a Y-maze trough. HPLC analyses demonstrated that TCA and TCD were the major bile acids found in bile, faeces, urine and tank

water from char. However, prespawning male urine also contained considerable amount of CA, CD, and an unidentified 3α-hydroxysteroid sulphate. Small quantities (<10%) of bile acids were sulphated at 3α-hydroxyl. Urinary bile acids in prespawning males were 100 times higher than in prespawning females. The amount of bile acids released by both prespawning males and females was sufficient to be detected by conspecifics. These data suggest that lake char have specific olfactory receptors with independent transduction mechanisms to detect and discriminate characteristic bile acid composition released by prespawning lake char. The perception of bile acids induces behaviour reactions. Thus, the results support the hypothesis that bile acids function as chemical signals in lake char.

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Finally, I dedicate this work to my parents and relatives in China.

List of Abbreviations

General abbreviations

B_{max}:	binding capacity
C-R:	concentration-response (or dose-response)
CSN:	ciliated sensory neuron
CV:	coefficient of variation
DDW:	deionized water, purified by MilliQ water system
DRN:	deciliated rosette tissue sections for non-specific binding
DRT:	deciliated rosette tissue sections for total binding
EEG:	electroencephalogram
EOG:	electroolfactogram
GD:	grain density
GL:	grey level
GV:	grey value of grains
HPLC:	high performance liquid chromatography
k':	relative capacity ratio
K_D:	dissociation constant
K_B:	dissociation constant for the adapting compound
MSN:	microvillar sensory neuron
NervX:	olfactory neuron degenerated tissue
NRN:	normal rosette tissue sections for non-specific binding
NRT:	normal rosette tissue sections for total binding
PGs:	prostaglandins
RGD:	relative grain density
RGI:	relative grey intensity
RL:	relative latency time
RR:	relative rising time
STD:	olfactory responses to 10^{-5} M L-serine
TPR:	tonic and phasic response ratio
UIS:	unidentified 3α-hydroxysteroid sulphate

Chemical abbrevations I--Bile acids

Abbrev.	Trivial Name	Chemical Name
ACA	Allocholic acid	3α,7α,12α-Trihydroxy-5α-cholan-24-oic acid
CA	Cholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid
CAS	Cholic acid 3,7,12-trisulphate	3α,7α,12α-Trihydroxy-5α-cholan-24-oic acid 3,7,12-trisulphate
CD	Chenodeoxycholic acid	3α,7α-Dihydroxy-5β-cholan-24-oic acid
CDM	Chenodeoxycholic acid diacetate methyl ester	3α,7α-Dihydroxy-5β-cholan-24-oic acid methyl ester 3,7-diacetate
CE	5β-Epicholestanol	3α-Hydroxy-5β-cholestane
CN	Cholanic acid	5β-Cholan-24-oic acid
DA	3-Deoxycholic acid	7α,12α-Dihydroxy-5β-cholan-24-oic acid
DC	Deoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid
DCS	Deoxycholic acid 3,12-disulphate	3α,12α-Dihydroxy-5β-cholan-24-oic acid 3,12-disulphate
DH	Dehydrocholic acid	3,7,12-Trioxo-5α-cholan-24-oic acid
EPZ	5β-Petromyzonol	3α,7α,12α,24-Tetrahydroxy-5β-cholane
GCA	Glycocholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid N-(carboxylmethyl)amide
GCD	Glycochenodeoxycholic acid	3α,7α-Dihydroxy-5β-cholan-24-oic acid N-(carboxylmethyl)amide
GDC	Glycodeoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid N-(carboxylmethyl)amide
GLC	Glycolithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid N-(carboxlymethyl)amide 3-sulphate
GLS	Glycolithocholic acid 3-sulphate	3α-Hydroxy-5β-cholan-24-oic acid N-(carboxlymethyl)amide 3-sulphate
HC	Hyocholic acid	3α,6α,12α-Trihydroxy-5β-cholan-24-oic acid
IDC	Isodeoxycholic acid	3β,12α-Dihydroxy-5β-cholan-24-oic acid
LC	Lithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid
LCS	Lithocholic acid 3-sulphate	3α-Hydroxy-5β-cholan-24-oic acid 3-sulphate

continued...

Chemcial abbreviations I--Bile acids (continued)

Abbrev.	Trivial Name	Chemical Name
NDC	Nordeoxycholic acid	23-Nor-3α,12α-dihydroxy-5β-cholan-24-oic acid
OCD	12-Oxochenodeoxycholic acid	3α,7α-Dihydroxy-12-oxo-5β-cholan-24-oic acid
ODA	3-Dehydrocholic acid	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid
ODC	7-Oxodeoxycholic acid	3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid
PZ	Petromyzonol	3α,7α,12α,24-Tetrahydroxy-5α-cholane
PZS	Petromyzonol sulphate	3α,7α,12α,24-Terahydroxy-5α-cholane 24-sulphate
TCA	Taurocholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TCD	Taurochenodeoxycholic acid	3α,7α-Dihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TCN	Taurocholanic acid	5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TCS	Taurocholic acid 3,7,12-trisulphate	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide 3,7,12-trisulphate
TDC	Taurodeoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TDH	Taurodehydrocholic acid	3α,7α12α-Trioxo-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
THD	Taurohyodeoxycholic acid	3α,6α-Dihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TLC	Taurolithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TLS	Taurolithocholic acid 3-sulphate	3α-Hydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide 3-sulphate
TOCN	Taurooxocholanic acid	3-Oxo-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TODC	Taurooxodeoxycholic acid	3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid N-(2-sulfoethyl)amide
TUC	Tauroursodeoxycholic acid	3α,7β-Dihydroxy-5β-cholan-24-oic acid
UC	Ursodeoxycholic acid	3α,7β-Dihydroxy-5β-cholan-24-oic acid
UCS	Ursodeoxycholic acid 3,7-disulphate	3α,7β-Dihydroxy-5β-cholan-24-oic acid 3,7-disulphate

Chemical abbreviations II--Chemicals other than bile acids

Abbrev.	Chemical Name
Amino acids	
ARG	L-arginine
CYS	L-cysteine
SER	L-serine
Prostaglandins	
15KPGF	15-Keto-prostaglandin $F_{2\alpha}$
13,14PGF	13,14-Dihydro-15-keto-prostaglandin $F_{2\alpha}$
PGF	Prostaglandin $F_{2\alpha}$
Gonadal Steroids	
17,20P	17α,20β-Dihydroxy-4-pregnen-3-one
17,20P-S	17α,20β-Dihydroxy-4-pregnen-3-one 20-sulphate
17,20,21P	17α,20β,21-Trihydroxy-4-pregnen-3-one
AG	3α,17β-Dihydroxy-5α-androstane 17β-glucuronide

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CHAPTER 1

General Introduction: Chemical Signals and Fish Behaviour

Living in an aquatic environment, a medium lacking in light but rich in dissolved compounds, many fish species have well-developed chemosensory systems to detect water-borne substances for information (chemical signals) to guide their social behaviours. Chemosensory systems include olfaction, gustation and common chemical senses (ref. Finger and Silver 1987; Hara 1992a). Each chemosensory system has its dedicated neural innervation and transduction elements. In the olfactory system, bipolar ciliated and microvillar sensory neurons are located in the olfactory epithelium of the nasal cavity and their axons form the olfactory nerve (cranial nerve I). The gustatory system is composed of taste buds containing receptor cells that are innervated by the facial (cranial nerve VII), glossopharyngeal (cranial nerve IX), or vagus (cranial nerve X) nerves. Common chemical senses are generally considered to be mediated by free nerve endings of general somatic afferent nerves (Parker 1912), although there is morphological evidence suggesting that nerve endings innervate scattered solitary sensory cells in the epidermis (ref. Whitear 1992). The definition of common chemical senses remains controversial (ref. Hara 1992b). More research is needed on the function of common chemical senses (ref. Silver 1987).

In fish, the role of olfaction in mediating physiological and behavioural responses has been studied more extensively than those of other chemical senses. The

olfactory perception of chemical signals has an indispensable effect on fish behaviours, including feeding, mating, kin recognition, homing and migration (ref. Hara 1982a, 1992a; Liley 1982). For example, research to date suggests that the olfactory system plays a dominant, if not exclusive, role in sex pheromone detection. Chemical cues released by reproductively mature fish may be detected by their partners -- sex pheromones. The perception of sex pheromones induces gonadotropin and steroid hormone elevation and consequent physiological changes, or stimulates spawning behaviour in these species. However, sex pheromones fail to induce similar reproductive physiological responses in anosmic individuals (Kobayashi et al. 1986; Stacey and Sorensen 1986; Dulka and Stacey 1991; Liley et al. 1993; Olsén and Liley 1993). Moreover, spawning behaviour is impaired in anosmic fish (Tavolga 1956; Partridge et al. 1976; Pollak 1978; Honda 1980; Stacey and Kyle 1983; van den Hurk and Lambert 1983; Kobayashi et al. 1986; Stacey and Sorensen 1986; Liley et al. 1993). Severance of goldfish olfactory tracts results in a decrease of gonadotropin level (Kim et al. 1995), which may affect reproduction. Pheromonal information is transmitted via the olfactory nerve, not the glossopharyngeal or vagus nerve, in the male three-spined stickleback *Gasterosteus aculeatus* (Segaar et al. 1983). Palatine nerve recordings in goldfish suggest a minimal role for the gustatory system in sex pheromone detection (Hara, per communication). The evidence also suggests that the terminal nerve, a cranial nerve that collocates with the olfactory system and innervates the base of olfactory lamellae, does not participate in the transmission of pheromonal information in goldfish (Kyle 1987; Fujita et al. 1991).

Sensory perception associates with electrical responses in the sensory system. However, the direct physiological evidence of olfactory responses to chemical stimuli was lacking until a pioneer report by Adrian and Ludwig (1938). They recorded electrical discharges from the olfactory tracts in catfish (*Ictalurus*) and several cyprinids when the olfactory organ was stimulated by watery extracts of fish foods, such as earthworms, liver tissues and blood. Electrical responses to olfactory stimuli have since been obtained from the fish olfactory epithelium, olfactory nerve and olfactory bulb (e. g. Gasser 1956; Shibuya 1960; Hara et al. 1965). It is now understood that, unlike olfaction in air-breathing vertebrates, the olfactory system of fish is specifically sensitive to non-volatile chemicals in the water. Currently, four major classes of chemicals have been considered as specific olfactory stimuli for fish. These stimuli are amino acids, prostaglandins (PGs), gonadal steroids and bile acids.

Amino acids

Amino acids are the most widely studied olfactory stimuli in fish. Electrophysiological studies have shown that detection thresholds for most stimulatory amino acids range from 10^{-10} M to 10^{-5} M in more than 30 species tested (ref. Hara 1992c, 1993, 1994a, b). Although different recording methods and experimental protocols make it difficult to compare the responsiveness, the general spectrum of olfactory responses to amino acids seems similar across fish species. Research to date indicates that olfactory responses to amino acids are mediated by at least two types of receptors, one for neutral and the other for basic amino acids (Brown and Hara 1981,

1982; Rhein and Cagan 1983; Rehnberg and Schreck 1986; Sveinsson and Hara 1990b). The presence of receptors specific for acidic amino acids is also suggested (Caprio and Byrd 1984, Caprio et al. 1989; Kang and Caprio 1991). Olfactory responses to amino acids in an agnathan species, sea lamprey *Petromyzon marinus*, appear quite different from those in other fishes. The olfactory system of sea lamprey is much more sensitive to basic amino acid L-arginine (ARG) and its derivatives than to neutral and acidic amino acids (Li and Sorensen 1992). Why lamprey narrowly tune to ARG-like amino acids is yet to be determined. Their nonparasitic larvae, ammocoetes, have similar olfactory response spectrum as adult lamprey (Zielinski et al. 1996). This suggests that olfactory specificity of sea lamprey to amino acids is not related to their parasitic living habit.

It is believed that the olfactory sensitivity to amino acids is closely associated with fish feeding behaviour, although it is also indicated that amino acids induce sexual behaviour and sperm release in the male rose bitterling *Rhodeus ocellatus ocellatus* (Kawabata et al. 1992a, b). By analysing the water that held the brook char *Salvelinus fontinalis* and brown trout *Salmo trutta*, Kleerekoper and Mogensen (1959) hypothesized that amino acids are the attractant compounds for sea lamprey in prey searching. Since then, there have been many reports regarding fish feeding reactions to amino acid-like compounds in food extracts (e. g. Hoese and Hoese 1967; Hashimoto et al. 1968; Sutterlin 1975; Carr and Chaney 1976; Pawson 1977; Adron and Mackie 1978; Johnstone 1980; Ellingsen and Døving 1986; Saglio et al. 1990; Løkkeborg et al. 1995). The chemical identity of these amino acid compounds has

been analysed. Glycine, betaine, alanine, proline and arginine are among the important amino acids that elicit food searching behaviour. In many cases, a mixture of amino acids is more effective than their individual components in inducing feeding behaviour. A mixture of amino acids that are inactive or even repulsive to fish, when applied individually, may become an effective feeding stimulant (Hashimoto et al. 1968; Adron and Mackie 1978; Saglio et al. 1990).

Behaviour responses to individual authentic amino acids vary among fishes. The coho salmon *Oncorhynchus kisutch* avoid 10^{-7} M threonine, alanine, serine or histidine, and are able to discriminate threonine from serine or alanine (Rehnberg and Schreck 1986). However, the Arctic char *Salvelinus alpinus* spend more time in water containing serine and alanine than in plain water (Jones and Hara 1985). Three patterns (attractive, repulsive and multi-model behaviours) are observed in the European glass eel *Anguilla anguilla* in response to amino acid stimulation, depending on background chemicals in the water and the concentration of stimulants (Sola et al. 1993). It would be interesting to determine if the different behaviour reactions to the same amino acid are simply due to species specificity.

The gustatory system of fish is also able to detect food stimuli in the surrounding water. Information is rare on which chemosensory system is responsible for, or how the olfactory and gustatory systems cooperate in food searching. Electric stimulation of olfactory tracts in the goldfish *Carassius auratus* and cod *Gadus morhua* induces food searching behaviours (Grimm 1960; Døving and Selset 1980). Occluding both fish nares, or bilaterally cutting olfactory tracts reduces food searching activity

(Parker 1910, 1911; Sheldon 1911; Olmsted 1918; Grimm 1960; Stacey and Kyle 1983). On the other hand, it has been reported that anosmic salmon are capable of discriminating food pellets in a way similar to normal fish (Sutterlin and Sutterlin 1970). Moreover, behavioural thresholds to amino acids are equivalent with or without the olfactory sense in the channel catfish *Ictalurus punctatus* (Holland and Teeter 1981). With the exception of L-proline, gustatory detection thresholds for amino acids are at least 2-3 logarithmic units higher than olfactory, when recorded from the palatine nerve in salmonids (Sutterlin and Sutterlin 1970; Marui et al. 1983; Hara et al. 1993, 1994). Behaviour responses to low concentration of amino acids in Arctic char are considered to be mediated by olfaction (Olsén 1986). It would be of interest to know if the synergistic effects of amino acid mixtures as feeding stimulants mentioned above are a consequence of participation by both olfactory and gustatory systems.

Prostaglandins

Prostaglandins are synthesized from fatty acid substrates within cell membranes. Generally, they function as hormones or second messengers in vertebrates (Hadley 1988). In fish, PGs are present in testes, ovaries, and blood (ref. Stacey and Goetz 1980). Administration of PGs induces female goldfish ovulation and sexual behaviour (Stacey and Pandey 1975; Stacey 1976, 1981; Sorensen et al. 1986). The emission of prostaglandin $F_{2\alpha}$ (PGF) and related compounds to the water from ovulated female goldfish functions as pheromones that stimulate male spawning

behaviour (Sorensen et al. 1988; Sorensen and Goetz 1993). Prostaglandins are also sex pheromones for the cobitid loach *Misgurnus anguillicaudatus* and Arctic char (Kitamura et al. 1994a; Ogata et al. 1994; Sveinsson and Hara 1995).

Prostaglandins are potent olfactory stimulants for many species. Of over 40 species tested electrophysiologically, 27 are sensitive to PGs. These 27 species belong to Cypriniformes, Salmoniformes, Osmeriformes (one species examined) and Gymnotiformes (one species examined) (Cardwell et al. 1992; Stacey et al. 1994; Kitamura et al. 1994a, b; Hara and Zhang 1996a). Olfactory detection thresholds for a few F_1, F_2 and E forms of PGs tested are below 10^{-7} M in goldfish (Sorensen et al. 1988), Arctic char (Sveinsson 1992) and two *Catostomus* species (Cardwell et al. 1992). Although many species can detect PGs at low concentrations, their olfactory sensitivities to PGs are species-specific, to some extent. For example, of the three most stimulatory PGs, PGF, 15-keto-prostaglandin $F_{2\alpha}$ (15KPGF) and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (13,14PGF), cobitid loach are more sensitive to 13,14PGF and 15KPGF, and goldfish to 15KPGF and PGF (Sorensen et al. 1988; Kitamura et al. 1994a), while Arctic char are less sensitive to 15KPGF than to PGF (Sveinsson 1992).

Gonadal steroids

Fish release considerable amounts of gonadal steroids into the water during the spawning season (Stacey et al. 1989; Van Der Kraak et al. 1989; Scott and Canario 1992; Scott and Liley 1994; Scott and Sorensen 1994; Scott et al. 1994; Sorensen and

Scott 1994). These gonadal steroids may function as sex pheromones (Colombo et. al. 1980; Stacey and Sorensen 1986; Sorensen et al. 1987; van den Hurk et al. 1987; Resink et al. 1989a, b, c; Stacey et al. 1989). Some of gonadal steroids are olfactory stimulatory at sub-nanomolar concentrations (Sorensen et al. 1987, 1995; Resink et al. 1989b; Moore and Scott 1992; Bjerselius and Olsén 1993; Irvine and Sorensen 1993). Olfactory responses to gonadal steroids are highly selective. In goldfish, the most extensively studied model, only 17α,20β-dihydroxy-4-pregnen-3-one (17,20P), 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20,21P), 4-androsten-3,17-dione and 4-pregnene-3,20-dione, and conjugated (both glucuronides and sulphates) 17,20P and 17,20,21P are stimulatory at nanomolar concentrations among over 60 gonadal steroids tested (Sorensen et al. 1987, 1990, 1992; Sorensen and Scott 1994). These compounds are also potent olfactory stimuli to 15 out of 19 species of Cypriniformes tested (Cardwell et al. 1992; Bjerselius and Olsén 1993; Irvine and Sorensen 1993; Stacey et al. 1994). However, salmonid species tested do not respond to 17,20P even at concentration of 10^{-7} M (Hara and Zhang 1996a; Chapter 2). Although their olfactory sensitivity to 17,20P is not determined by electrophysiological means, precocious male chinook salmon *Oncorhynchus tshawytscha* avoid water scented with 17,20P at 10^{-8} M (Dittman and Quinn 1994). This behaviour reaction is similar to the observations in spermiated goldfish and crucian carp *Carassius carassius* (Bjerselius et al. 1995a, b). Different gonadal steroids may function as sex pheromones in other species. For example, 3α-hydroxy-5β-androstan-17-one 3-glucuronide, 3α,17α-dihydroxy-5β-pregnane-20-one 3-glucuronide and 11β,17β-dihydroxy-4-androstene-3-

one are pheromone-like compounds for the black goby *Gobius jozo* (Colombo et. al. 1980), the African catfish *Clarias gariepinus* (Resink et al. 1989a, b, c) and the yellowfin Baikal sculpin *Cottocomephorus grewingki* (Katsel et al. 1992), respectively.

Recent studies suggest that 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate (17,20P-S) is another potent pheromonal steroid for goldfish (Sorensen et al. 1991b, 1995; Scott and Sorensen 1994). Its primer pheromonal function, which stimulates gonadotropin release and sperm production in the male partner, may be similar to that of 17,20P in goldfish. The olfactory response to 17,20P-S is mediated by a receptor different from that for 17,20P (Sorensen et al. 1995).

The biological significance of 17,20P-S in species other than goldfish is largely unknown. Large quantities of 17,20P-S are found in the urine of the female Atlantic salmon *Salmo salar* and Dover sole *Solea solea*, and of both genders of the plaice *Pleuronectes platessa* and rainbow trout *Oncorhynchus mykiss* (Scott and Canario 1992; Moore and Scott 1992; Scott and Liley 1994). A surge in urinary excretion of 17,20P-S in female rainbow trout coincides with the time of ovulation (Scott and Liley 1994). However, 17,20P-S has little priming effects on circulating hormonal levels. It alters neither the orientation behaviour nor the spawning activity of ripe male rainbow trout. These results contrast sharply with the effects of crude urine from ovulated or unovulated rainbow trout (Scott et al. 1994). Urine of mature female rainbow trout contains substances that have primer pheromonal functions.

The only salmonid species that olfactory responses to 17,20P-S have been examined is Atlantic salmon. It was found that only precocious males may respond to

17,20P-S. The olfactory system becomes sensitive to 17,20P-S only after being exposed to urine from ovulated females (Moore and Scott 1992). This olfactory facilitation happens in minutes. The mechanism of this quick activation is unknown. Olfactory responses to some gonadal steroids show gender differences during the spawning season in Atlantic salmon (Moore and Scott 1991), crucian carp (Bjerselius and Olsén 1993) and goldfish (Bjerselius and Olsén 1993; Irvine and Sorensen 1993). The olfactory activity could be altered 1-3 weeks after gonadal steroid treatments (Hara 1967; Oshima and Gorbman 1968, 1969; Cardwell et al. 1995). However, judging from the time course of olfactory facilitation in precocious Atlantic salmon (in 3 min), it is unlikely to involve the formation of new receptors. A possible explanation would be that unknown compounds from ovulated female urine have temporarily removed inhibitory factors or changed membrane properties of specific neurons responsive to 17,20P-S.

Bile acids

Bile acids are another type of steroid naturally produced and excreted by fish. They are essential for food digestion and various aspects of organic and anionic transport in the vertebrate digestive system. One of the most interesting features is that animals at similar systematic positions tend to have similar bile acids (Haslewood 1967a, b, 1978; Tammar 1974). A wide variety of bile acids in vertebrates is enigmatic, for the composition of bile acids from any species is, in principle, able to function perfectly as a digestive aid. Moreover, the bile acid composition is not

related to feeding habits (Haslewood 1978). However, the diversity of bile acid composition among animal groups may become important if bile acids serve as chemical signals in vertebrate. Indeed, the pheromonal role of bile acids in lactating rat has been indicated (Moltz and Leidahl 1977; Kilpatrick et al. 1979).

The role of bile acids as chemical signals has been an interesting topic in fish chemoreception since the report by Døving and his coworkers (1980). Using electroencephalogram (EEG) recording, they found that bile acids are potent olfactory stimulants for the grayling *Thymallus thymallus* and Arctic char. Subsequently, it was discovered that bile acids are also the most potent fish gustatory stimuli ever found (Hara et al. 1984). Taurocholic acid (TCA) is a potent olfactory stimulus to several salmonids (Hara et al. 1984; Quinn and Hara 1986; Zhang and Hara 1991; Hara and Zhang 1996a, b), goldfish (Sorensen et al. 1987), white sucker *Catostomus commersoni*, and longnose sucker *C. catostomus* (Cardwell et al. 1992). Bile acids are considered less stimulatory than amino acids for channel catfish when comparing response magnitudes at 10^{-4} M (Erickson and Caprio 1984). In the zebrafish *Danio rerio*, olfactory response magnitudes to some bile acids and amino acids are higher in females than in males at high concentrations (Michel and Lubomudrov 1995).

Information on bile acid excretions in fishes is limited. Anadromous Arctic char release 34 nmol of 3α-hydroxysteroids per kilogram body weight into the water every hour (Selset 1980; Døving et al. 1980). Sea lamprey larvae excrete three uncommon bile acids, allocholic acid (ACA), petromyzonol (PZ) and petromyzonol sulphate (PZS) (Li et al. 1995). Natural excretory bile acids may provide a constant

source of population- or species- specific odours for kin recognition and homeward migration in salmonids and sea lamprey (Dϕving et al. 1980; Selset 1980; Selset and Dϕving 1980; Stabell 1987; Li et al. 1995). Behavioural studies have demonstrated that Arctic char and Atlantic salmon are attracted to juvenile intestinal contents (Selset and Døving 1980; Olsén 1987; Stabell 1987). A low concentration of TCA could induce fish preference behaviour (Jones and Hara 1985), or snapping behaviour (Hellstrøm and Døving 1986). However, some bile acids are repellent for European glass eels at high concentrations (Sola and Tosi 1993).

Bile acids as chemical signals in lake char

The lake char *Salvelinus namaycush* is one of the most important commercial and sports fishes in North America. Lake char populations in the Great Lakes were severely damaged during late 1950s and early 1960s, largely because of excessive fishing and parasitic sea lamprey ravage (Swanson and Swedberg 1980; Coble et al. 1990). Restoration of self-sustaining stocks of lake char throughout the Great Lakes is a long term goal for the North American fisheries industry. Despite massive plantings of yearling lake char in Lake Superior during 1960-1970, there is little evidence that the natural reproduction is directly attributable to the planted fish (Olver and Lewis 1977; Brown et al. 1981; Swanson 1982). It has been suggested that lake char home to their natal reef to spawn (Eschmeyer 1955, Loftus 1958; Rahrer1968; Martin and Olver 1980; Horrall 1981). The lack of some natal environment elements in Lake Superior may account for the failure of planted lake char to spawn (Martin and Olver

1980; Horrall 1981; Brawn et al. 1981; Goodier 1981). Studies have shown that artificial spawning beds with rocks and rubble fail to attract spawning lake char. However, sites with penned ripe fish, seeded with eyed eggs, and rocks from used spawning areas, are used by lake char as spawning grounds (Martin 1960; Paterson 1968). Under laboratory conditions, Foster (1985) found that ripe lake char chose a place with abundant faeces from their young-of-the-year as spawning site. This suggests that nocturnal spawning lake char might rely on bile acid-like substances to locate spawning sites and partners, and to synchronize spawning behaviour.

To investigate pheromonal roles of bile acids in lake char, my graduate research has focused on: 1) how bile acids are detected by olfaction, 2) what kinds of bile acids are produced and released by char, and 3) whether bile acid signals affect char behaviour. To answer these questions, I have used electrophysiological methods to examine olfactory sensitivity and specificity of lake char to bile acids (Chapter 2). I have characterized and localized bile acid receptors in the olfactory epithelium using cross-adaptation methods and autoradiographic binding assays (Chapter 2, 3). The data suggest that lake char olfactory responses, as well as behavioural preference, to conspecific bile are associated with bile acid components (Chapter 4). HPLC analyses of extracts from char bile, faeces, urine and tank water have revealed that bile acids are mainly released via faeces, urine and possibly some other routes into the water. The bile acids lake char released are among the most potent for olfactory stimulation. The composition and quantitative changes of bile acid excretion by lake char during the spawning season suggest the biological significance of bile acids in reproduction

(Chapter 5). My studies, from the aspect of bile acid release, sensory receptor detection and chemical signal perception, have provided substantial evidence to support the hypothesis that bile acids function as pheromones in lake char.

CHAPTER 2

Electrophysiological Characterization of Olfactory Receptors for Bile Acids

INTRODUCTION

Odour recognition in vertebrates is accomplished by a large number of different receptors in sensory neurons in the olfactory epithelium (Buck and Axel 1991; Ngai et al. 1993a, b, c). Over the last several decades ample evidence has accumulated that the detection and discrimination of distinct odorants result from the association of odorous ligands with specific receptors on olfactory neurons (e.g. Amoore et al. 1972; Lancet 1986; Buck and Axel 1991; Ngai et al 1993a, b, c). This interaction leads to electric voltage changes on the membrane of olfactory neurons, which can be readily recorded electrophysiologically. Electroolfactogram (EOG) recording is widely used for screening the olfactory sensitivity to stimulants, as the EOG represents voltage transients of sensory neurons across the surface of the olfactory epithelium in response to chemical stimulation (Ottoson 1956, 1974; Getchell 1974). In this study, I use the EOG recording to examine olfactory responses of lake char to bile acids.

Despite growing interest in the role of bile acids in fish chemoreception (ref. Chapter 1), little effort has been made to examine structure-activity relationships and

receptor specificity for bile acids in the fish olfactory system. This hampers our understanding on how bile acids are detected by fish. Analysis of structure-activity relationships will reveal the relative effectiveness of stimulants interacting with the putative receptors and consequently provide information on whether a particular stimulus will be effectively detected by fish and which structural features are required for a stimulant to interact with the receptors.

Other common strategies to physiologically characterize olfactory receptors are cross-adaptation and mixture experiments. Like other sensory receptors, olfactory receptors encounter fatigue or adaptation to a certain degree during exposure to one type of stimulus, while the receptive ability of other receptors remains almost unaffected. Therefore, cross-adaptation may be used to investigate the involvement of a single or multiple receptors mediating an observed electrophysiological response. By exposing the receptor to chemical A at a saturable concentration for a prolonged period of time, followed by stimulation with chemical B, the response to chemical B obtained is considered to be mediated by receptors and/or transduction mechanisms distinct from those for chemical A (Limbird 1986). Furthermore, if chemicals A and B are mediated by separate receptors, stimulation with a mixture of A and B should result in a summation of the responses induced individually. Thus, mixture experiments may provide clues on whether the compounds in the mixture interact with different types of receptors and whether suppression and synergism of the responsiveness to a mixture stimulation occur at the receptor level.

When characterizing a chemosensory receptor in studies using

electrophysiological means, the terms "receptor site", "receptor type" and "receptor process" have been used interchangeably with "receptor" or "transduction mechanism" (Hara 1977, 1982b; Caprio and Byrd 1984; Ohno et al. 1984). "Receptor" will be used here to denote the postulated specific molecular sites or proteins on olfactory membranes with which a stimulus must react in order to elicit characteristic responses and all subsequent events leading to production of the generator potential in olfactory neurons.

In this chapter, I will first analyse the characteristics of EOG response features to bile acids as compared to those to amino acids. I will then focus on studies of olfactory sensitivity and specificity to bile acids by analysing structure-activity relationships, and by using cross-adaptation and binary mixture methods. Finally, I will examine the olfactory sensitivity to bile acids at different life stages of lake char.

MATERIALS AND METHODS

Experimental animals

Lake char eggs were obtained from the Experimental Lakes Area, Ontario, and Clear Lake, Saskatchewan, Canada. They were hatched in the Freshwater Institute, Department of Fisheries and Oceans, Canada. Eggs were incubated at 5.5-6.5°C, in baskets made of plastic mosquito screen on fibreglass frames and suspended under the surface of a flowing water, until the yolk was absorbed. Lake char were then

maintained with commercial food pellets and supplied with flowing, aerated dechlorinated Winnipeg city water (plain water) at 11.5-12.5°C under 16L:8D photoperiod. Mature fish were under an artificial seasonal light cycle (Poll-Branscheid and Holtz 1990). Experimental procedures employed in the studies described in Chapter 2-5 complied with the guidelines issued by the Canadian Council for Animal Care.

EOG recording

Each fish was tranquilized with MS222 (1:8000), anaesthetized intraperitoneally with amobarbital (30 mg/kg body weight), and immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide, 3-4 mg/kg body weight). The anaesthetized fish was wrapped with an absorbent tissue and secured in a holding apparatus placed in a plexiglass trough. The right side rosette was exposed by removing the dorsal aspect of the skin and the cartilage of the olfactory sac. The naris and gills were perfused with plain water. To deliver plain water or stimulants to the naris, the method of Sveinsson (1992) was used. Briefly, a pneumatic activator switches plain water to/from the stimulant solution delivered by two identical polyethylene tubes. The pneumatic valve was controlled by a solenoid and an associated electronic timing device (Hara et al. 1973). The solution flowed through a glass capillary positioned over the rosette (10 ml/min). By using this stimulation system, it is possible to achieve an approximate square stimulation at required concentration each time with no apparent interruption or disturbance of the flow to the

naris. The stimulus duration was 10 s. A time delay from onset of the switch to the stimulus reaching olfactory epithelia was 0.6-0.7 s.

EOG responses were recorded differentially by a pair of Ag-AgCl electrodes (type MEH1S; World Precision Instruments, FL, USA) filled with 3 M KCl bridged by 8% gelatin-saline filled capillaries (tip diameter 100-150 μm) (Evans and Hara 1985). One electrode was positioned near the central ridge, posterior portion of the rosette, and slightly above the olfactory epithelium. The other (reference) electrode was placed on the skin surface adjacent to the perfused olfactory cavity. Electrical activities were amplified with a DC-preamplifier (Grass 7P1, Grass Instruments, MA, USA), digitized by a storage oscilloscope (Model 2220, Tektronix Canada, AB, Canada), and analysed on-line with a microcomputer using Tektronix GURU II hardware/software utilities (Sveinsson 1992).

Multi-unit recording

From embryo to alevin periods, olfactory responses of lake char to chemical stimulation were monitored using multi-unit recording. Developmental stages were assigned according to Balon (1980). The recording procedures were basically the same as those described by Zielinski and Hara (1988). Briefly, the embryo was anaesthetized by MS 222 after the egg capsule was removed. The anaesthetized embryo was secured onto a ramp moulded from paraffin in a plexiglass trough. A consistent water flow was maintained throughout the experiments. During the stimulation, plain water was replaced by chemical solution. The clearance of water in

the trough took about one half minute.

Tungsten microelectrodes (tip diameter, 0.025 cm) insulated with Parylene (A-M System, WA, USA) were used for recording. Electrical signals were amplified with an A. C. amplifier (Grass P511), integrated and displayed on an oscilloscope.

Stimulus administration and experimental protocol

Stock solutions were prepared with distilled water and stored in a refrigerator. Concentrations of stock solutions were 10^{-2} M for amino acids and 10^{-4}-10^{-3} M for bile acids, depending on the solubility. Test solutions were formulated immediately before testing by diluting with plain water. To eliminate the effect of distilled water, stock solutions were diluted with at least 100 times of plain water to form test formulas. Bile acids tested and their abbreviations are listed in Table 2.1. Trivial names were used. Names of some uncommon bile acids complied with the nomenclature guides proposed by Hofmann et al. (1992). Abbreviations followed a rule of thumb; an "S" at the end indicates the sulphated forms; a "T" or "G" at the beginning indicates taurine or glycine amidated forms, respectively; and an "O" at the beginning indicates an additional oxo group is attached to the sterol rings of a "parent" bile acid. The term "bile acid" in this paper includes all compounds having the cholanoate structure and does not solely denote the protonated acid form. A schematic structure of the bile acid represented by cholic acid (CA) is shown in Figure 2.1, whose legend briefly explains bile acid nomenclature and typical substituents.

Cholic acid 3,7,12-trisulphate (CAS), deoxycholic acid 3,12-disulphate (DCS),

Table 2.1 Detection thresholds of olfactory responses to bile acids tested.

Trivial Name	Abbrev.	Thresholds (nM)
Chenodeoxycholic acid	CD	0.02
3-Deoxycholic acid	DA	0.02
3-Dehydrocholic acid	ODA	0.02
Taurolithocholic acid 3-sulphate	TLS	0.02
Cholic acid	CA	0.1
Taurocholic acid 3,7,12-trisulphate	TCS	0.1
Hyocholic acid	HC	0.2
Taurochenodeoxycholic acid	TCD	0.5
Taurocholic acid	TCA	0.5
7-Oxodeoxycholic acid	ODC	1
Petromyzonol sulphate	PZS	1
Lithocholic acid 3-sulphate	LCS	2
12-Oxochenodeoxycholic acid	OCD	2
Taurodeoxycholic acid	TDC	2
Deoxycholic acid	DC	2
Isodeoxycholic acid	IDC	5
Glycolithocholic acid 3-sulphate	GLS	5
Allocholic acid	ACA	10
Glycocholic acid	GCA	10
Lithocholic acid	LC	10
Petromyzonol	PZ	20
5β-Petromyzonol	EPZ	20
Glycochenodeoxycholic acid	GCD	20
Taurolithocholic acid	TLC	20
Deoxycholic acid 3,12-disulphate	DCS	50
Glycodeoxycholic acid	GDC	50
Ursodeoxycholic acid 3,7-disulphate	UCS	100
Taurohyodeoxycholic acid	THD	100
Tauroursodeoxycholic acid	TUC	100
Dehydrocholic acid	DH	200
Cholic acid 3,7,12-trisulphate	CAS	200
Cholanic acid	CN	200
Nordeoxycholic acid	NDC	200
Glycolithocholic acid	GLC	500
Taurodehydrocholic acid	TDH	500
Taurocholanic acid	TCN	1000
Chenodeoxycholic acid diacetate methyl ester	CDM	1000
5β-Epicholestanol	CE	2000

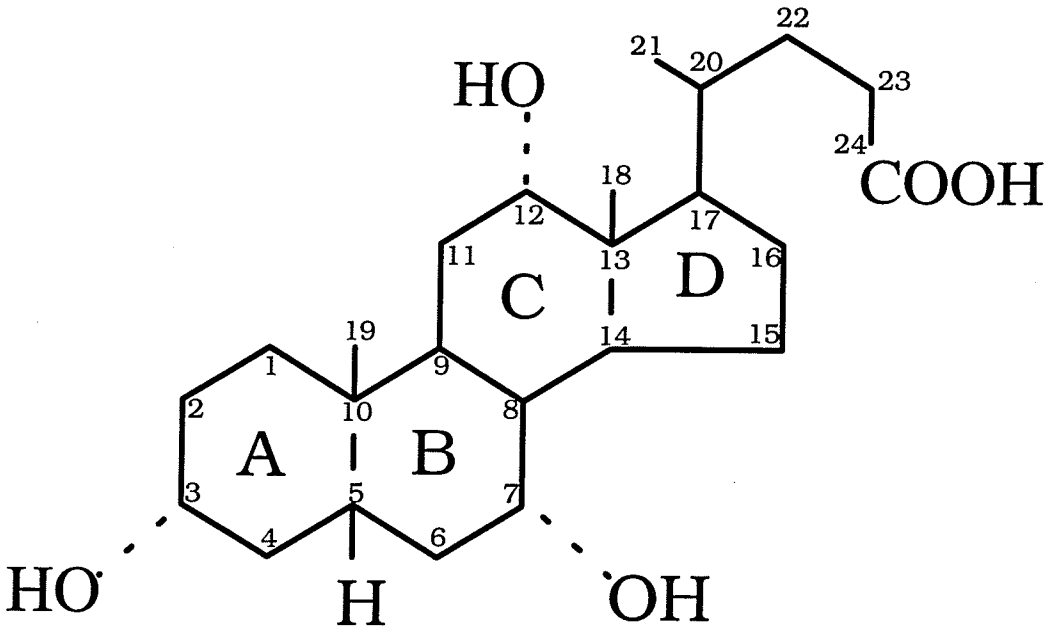


Figure 2.1 Structural formula of cholic acid, a typical bile acid in vertebrates. A 24-carbon bile acid without any hydroxylic substituents is termed cholanic acid, composed of a four-ring cyclopentanophenanthrene nucleus, with two methyl groups between rings at the position 18 and 19, and a 5-carbon carboxylic acid side chain attached to C_{17} position. Bile acids having 4-carbon side chain are *nor* cholanic acids, and having 7-carbon side chain are cholestanic acids. Cholane and cholestane are used in the systematic name for the bile acid skeleton with methyl terminal of the side chain. The orientation of the hydrogen atom at the C_5 position determines stereochemistry of the ring junction. Cholic acid, as many other bile acids, is A/B *cis* juncture (5β- configuration). The 5α- bile acids are termed *allo* bile acids. In 5α-configuration, A, B, C and D rings are virtually in the same plane. In 5β-configuration, B, C, and D rings are in the same plane, while the A ring bends away and forms a kink which makes the nucleus in "⌐" shape. Most bile acids have one or more hydroxyls attached on sterol rings. These hydroxyls are either equatorial (in the plane) or axial (out of the plane) orientated. In 5β- bile acids, the 3α- and 6α-hydroxyls are placed in equatorial and the 7α- and 12α- hydroxyls in axial. The difference of hydroxyl orientation between bile acids and allo bile acids is that the 3α-hydroxyl in the latter is axial. Bile acids may make side chain N-acyl conjugation with taurine or glycine (amidation), or conjugated with sulphate ester at hydroxyl (sulphation), or both (Carey 1985; Hofmann et al. 1992).

ursodeoxycholic acid 3,7-disulphate (UCS) and taurocholic acid 3,7,12-trisulphate (TCS) were gifts from Dr. Ibrahim Yousef, Department of Pharmacology, University of Montreal, Canada, and ACA, PZ and PZS were from Dr. Peter Sorensen, Department of Fisheries and Wildlife, University of Minnesota, USA. All other chemicals, with the purest form available, were purchased from either Steraloids, NH, USA or Sigma Chemical, MO, USA.

The experimental protocol was based on Sveinsson and Hara (1990a) with minor modifications. Each chemical was examined in order from low to high concentrations. A control stimulus (plain water in a testing beaker) was applied at the beginning of each concentration series. Each concentration was tested once with a 3-min interval. L-Serine at 10^{-5} M was tested at the beginning and the end of each test period as the standard response (STD). The magnitude of the phasic response was designated as the maximum height displacement from the baseline and the tonic response was measured from the plateau at the end of stimulation to the transient minimum during the baseline reinstatement. If the magnitude of STD had changed more than 30% during a period, the data of this period were considered inconsistent and dismissed from analysis. The data were normalized as relative magnitudes of STD at each period and presented as means obtained from at least three fish. The electrophysiological threshold mentioned here was defined as the lowest test concentration that elicited a definite response larger than that of the control stimulation.

For cross-adaptation, the original response -- the response before adaptation --

was first obtained as mentioned above, and then the adapting solution was applied to perfuse the olfactory rosette throughout adaptation tests. Adaptation tests began at 10 min after adaptation started to ensure the olfactory epithelium had reached a steady state of adaptation. A baseline activity response, when the adapting solution in the testing beaker was delivered to the naris, was an indication of proper acclimation. The stimulant was first diluted with distilled water to a concentration series. The test formula during adaptation period was made by 1000 times dilution using the adapting solution instead of plain water used in original response tests. The background response to the control stimulus was subtracted from the response magnitude in each test period.

The concentration-ratio (or dose-ratio) equation was adopted to measure whether bile acids interact with their receptors in a truly competitive manner when the full agonist exists. The calculation was based on the equation described by Tallarida and Jacob (1979). The dissociation constant for the adapting compound (K_B) should be constant when adaptation is applied at different concentrations, if the adapting compound behaves the same as the test compound. A constant K_B thus also indicates C-R curves shift in a parallel pattern.

In mixture experiments, the choice of concentrations for stimulation was based on the results of concentration-response (C-R) relationship of stimulants in questions. According to the ligand-binding theory (ref. Boeynaems and Dumont 1980; Limbird 1986), if there were receptors *a* and *b* that are specific for chemicals A and B, respectively, and chemical A may compete with chemical B for the receptor *b* due to

the similarity of molecular structures between A and B, chemical A should have the highest affinity to its own receptor *a* first. The "over-dosed" molecules would interact with the next highest affinity receptor (e.g receptor *b*) after receptor *a* was totally occupied. Thus, relatively low concentrations that elicit moderate responses were chosen in the present study. When examining a nonstimulatory chemical for antagonistic effects, a relatively high concentration was administered to ensure possible antagonistic effects would be clearly observed. Equipotent stimulus mixtures, as proposed by Caprio et al. (1989), were unnecessary for this study.

EOG feature analysis

To examine characteristics of EOG features in response to bile acids, as compared to those to amino acids, an EOG waveform was divided into four phases: 1) latency phase, from the onset of stimulus delivery to the beginning of the response, 2) rising phase, from the beginning of the response to a maximum height reached, 3) falling phase, from the peak amplitude to the end of stimulation, and 4) recovery phase, from the cessation of stimulation until a steady state of baseline was achieved (Fig.2.2). With the present recording system, it is possible to measure transient voltage changes with an accuracy on the order of a 0.1 s period. The time of latency phase, the time of rising phase, and the tonic/phasic response ratio (TPR) were used to compare EOG features for two classes of stimuli. Comparison was made at the equivalent magnitude as that of STD at the same test period, because the elements to be compared are more closely correlated with the response magnitude than a particular

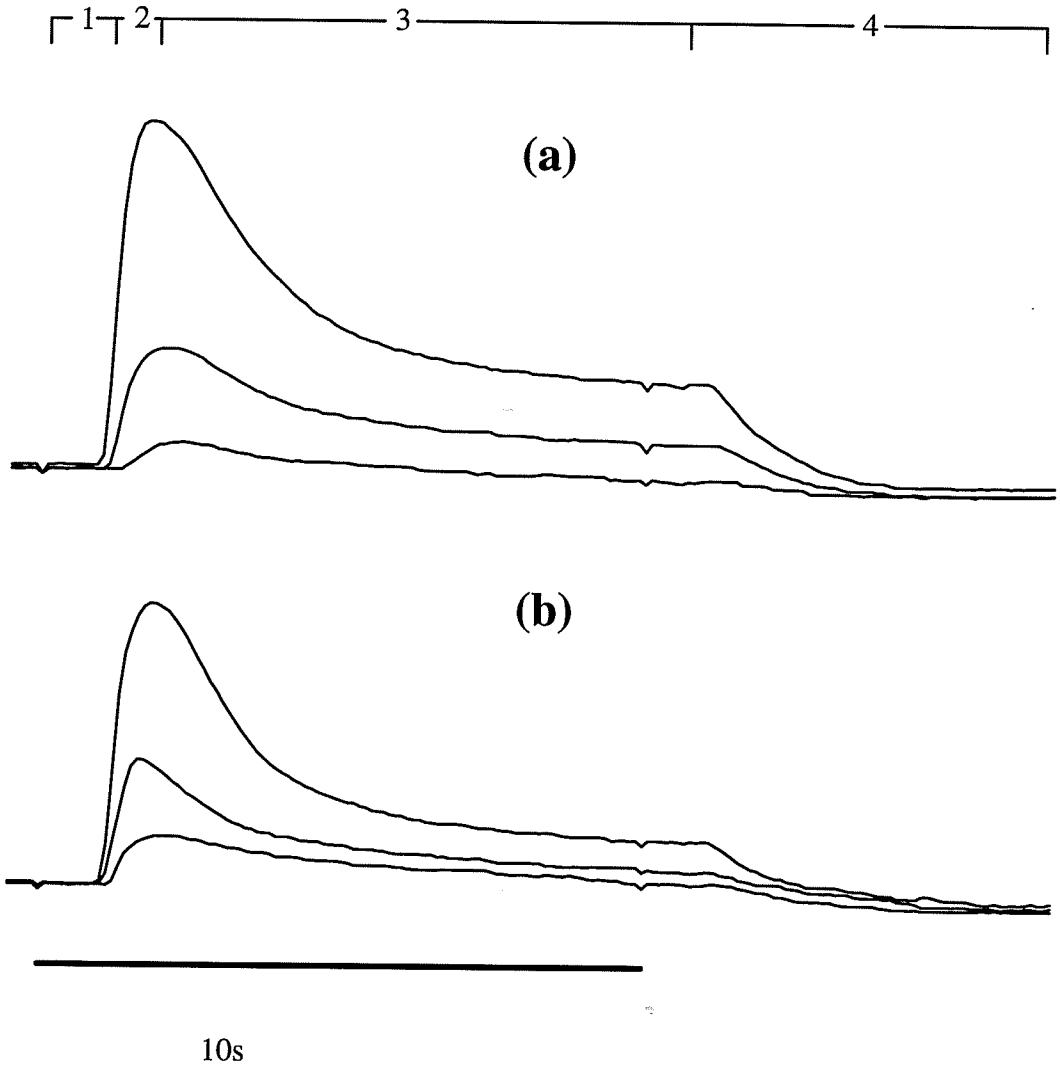


Figure 2.2 Traces of electroolfactogram responses to **(a)** L-cysteine and **(b)** taurocholic acid at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M. Top ticks designate ranges of the four phases, latency phase (1), rising phase (2), falling phase (3), and recovery phase (4). Refer to the text for the definition of the four phases.

concentration for a given compound (e. g. coefficient of variation CV=5.5% *vs* 7.5%, n=22). All EOG waveforms met the above requirements were included for analysis. However, due to relatively low response magnitudes for many bile acids (see Results section), data analysed are actually from olfactory responses to TCA, taurochenodeoxycholic acid (TCD), taurodeoxycholic acid (TDC) and taurolithocholic acid 3-sulphate (TLS). Data for amino acids are from olfactory responses to L-cysteine (CYS), L-arginine (ARG) and L-serine (SER). To avoid any possible variation caused by factors other than the stimulation, the relative latency time (RL) and relative rising time (RR) were introduced. The RL was defined as the time of latency phase of an individual response minus that of STD at the same test period, and the RR was the time of rising phase of an individual response minus that of STD at the same test period.

Scanning electron microscopy

To examine whether olfactory responses of embryonic lake char are associated with the sensory neuron differentiation, the morphology of olfactory sensory neurons in developing lake char embryos was examined by means of scanning electron microscopy. Heads of embryonic char were fixed with 0.025 M phosphate buffer containing 5% glutaraldehyde (pH 7.2). After stage $E^4$7, specimens were fixed with 5% glutaraldehyde in 0.05 M phosphate buffer. The above fixative solution was refreshed once during 4 hrs of fixation. The specimens were then subjected to 1% osmium tetroxide treatment for 2 hrs. After rinsing with distilled water, dehydrating

in a graded series of ethanol, and drying at the critical point in a Sorvall apparatus, the dried specimens were mounted on metal stubs using conducting silver paint and coated with gold in a sputter device. The specimens were examined under a Cambridge Stereoscan scanning electron microscope (Zielinski and Hara 1988).

Statistics

The data presented in this chapter are means and standard errors. One-way ANOVA and Tukey's HSD tests were used for multiple comparisons (SYSTAT program, Systat, IL, USA). Student t-test was used for two pair variable comparison. Probability $P < 0.05$ was considered significantly different.

RESULTS

Characteristics of EOG responses

When bile acid stimulants reached the olfactory epithelium, negative monophasic EOG responses were monitored. The general shapes of EOG responses for bile acids were similar to those for amino acids (Fig. 2.2). Positive voltage changes were not observed. Generally, response magnitudes for bile acids varied proportionately to those for amino acids at any regions in the epithelium where the electrode was placed. The EOG response rose from the baseline to its peak amplitude 1-2 s after the stimulation switch was turned on. Then, the waveform underwent an exponential decline to a maintained tonic component throughout the 10 s stimulation.

After cessation of the stimulation, the potential dropped quickly to half of the tonic magnitude in about 1.5 s. All components of an EOG waveform were associated with the response magnitude for a given compound. As the stimulant concentration increased, the time of latency phase became gradually shorter, whereas the time of recovery phase became longer. At the response magnitude equivalent to that of STD, there was an overall 0.3-0.6 s response delay after stimuli reached the epithelium; the period of the rising phase were 0.6-1.2 s, with an average of 0.87 ± 0.02 s ($n=80$).

Table 2.2 shows that RL was longer for responses to bile acids as compared to that to amino acids, while the RR was not significantly different between responses to bile acids and amino acids. In addition, the restoration from tonic plateau to baseline was slower for bile acids.

The magnitude of both phasic and tonic components rose with the increase of stimulus intensity. The TPRs were inversely related to stimulant concentrations and became constant at high concentrations (Fig. 2.3). The declination in TPR curves along with the increase in concentrations was fastest for taurolithocholic 3-sulphate (TLS) and slowest for chenodeoxycholic acid (CD) among three representative bile acids. The TPRs of EOG responses were not similar among various bile acid stimulations or among various amino acid stimulation, when compared at equivalent response magnitude as that of STD ($P > 0.4$). The TPRs of EOG responses, however, differed highly significantly between bile acid and amino acid stimulation ($P < 0.002$) (Table 2.3).

Table 2.2 Comparison of relative latency time (RL) and relative rising time (RR) between olfactory responses to bile acids (BA) and amino acids (AA). Refer to the text for the definition of RL and RR.

	Relative Latency Time (s)		Relative Rising Time (s)	
	RL-BA	RL-AA	RR-BA	RR-AA
Mean±SEM	0.09±0.015	0.02±0.017	0.11±0.030	0.14±0.026
N	19	19	12	12
t-test	$P < 0.005$		$P > 0.4$	

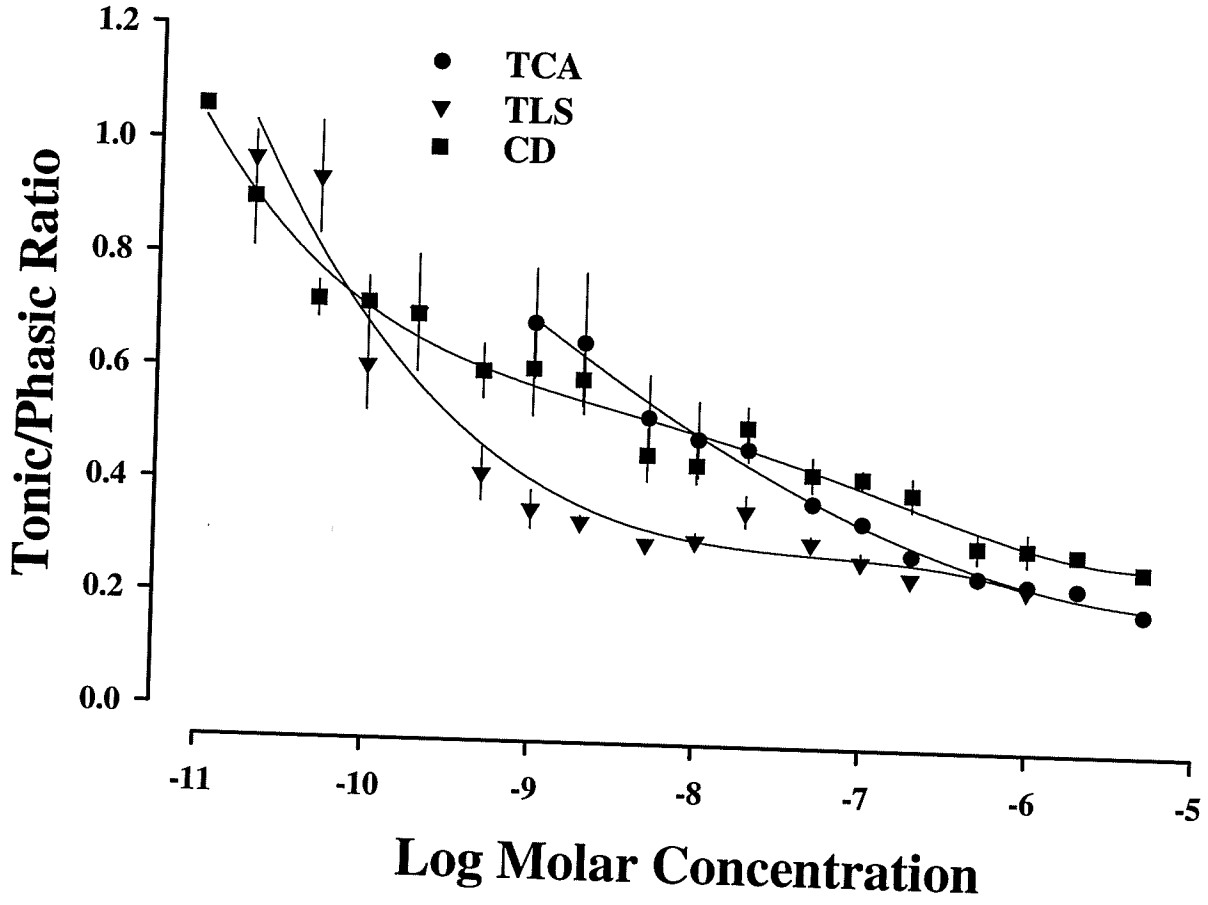


Figure 2.3 Tonic/phasic response ratios for three representative bile acids at various concentrations. TCA=taurocholic acid, TLS=taurolithocholic acid 3-sulphate, CD=chenodeoxycholic acid.

Table 2.3 Tonic/phasic response ratios (TPRs) of electroolfactogram responses to several bile acids and amino acids when compared at equivalent response magnitudes as those of responses to 10^{-5} M L-serine.

Chemicals[a]	N	TPRs (Mean±SEM)	Statistics[b]	
TCA	9	0.213±0.011	A	
TCD	6	0.237±0.007	A	
TDC	7	0.230±0.014	A	
TLS	6	0.227±0.012	A	$P_{A/B} < 0.002$
ARG	9	0.317±0.010	B	
CYS	9	0.304±0.012	B	
SER	9	0.347±0.021	B	

[a] ARG=L-arginine; CYS=L-cysteine; SER=L-serine; TCA=taurocholic acid; TCD=taurochenodeoxycholic acid; TDC=taurodeoxycholic acid; TLS=taurolithocholic acid 3-sulphate.

[b] TPRs denoted by the same letter (A or B) are not different from each other ($P > 0.4$).

Olfactory sensitivity and concentration-response relationships

Bile acids with various substituents were tested for effectiveness as olfactory stimulants. Olfactory response thresholds for 38 bile acids tested are listed in Table 2.1. The lowest detection threshold was 0.02 nM. Nine bile acids had detection thresholds at sub-nanomolar concentrations. The relationships between magnitude of phasic response and stimulant concentrations for 30 bile acids are shown in Figure 2.4. Response magnitudes for all bile acids tested showed a concentration dependent pattern.

All free bile acids showed similar concentration-response (C-R) curves; a plateau lasts about 2 logarithmic unit concentrations after a semi-logarithmic linear increase at first 2 logarithmic unit concentrations, followed by another fast increase at higher concentrations (Fig. 2.4a, b).

Of free bile acids, CD, 3-deoxycholic acid (DA), 3-dehydroxycholic acid (ODA), CA and hyocholic acid (HC) were the most potent, with detection thresholds of 0.02-0.2 nM. These acids have an α-hydroxyl at C_7 or C_{12}, or both. Replacing an hydroxyl with an oxo group at either C_7 or C_{12} reduced stimulatory effectiveness by about 1 logarithmic unit (OCD or ODC *vs* CA). The absence of hydroxyls or the presence of oxo groups instead of hydroxyls, as in cholanic acid (CN) and dehydrocholic acid (DH), diminished the stimulatory effectiveness. An hydroxyl at C_3, however, was marginally important and might improve the stimulatory effectiveness when it was the only hydroxy substituent (LC *vs* CN). The oxo substituent at C_3 position had no effects (ODA *vs* DA). Configuration of α- or β- in

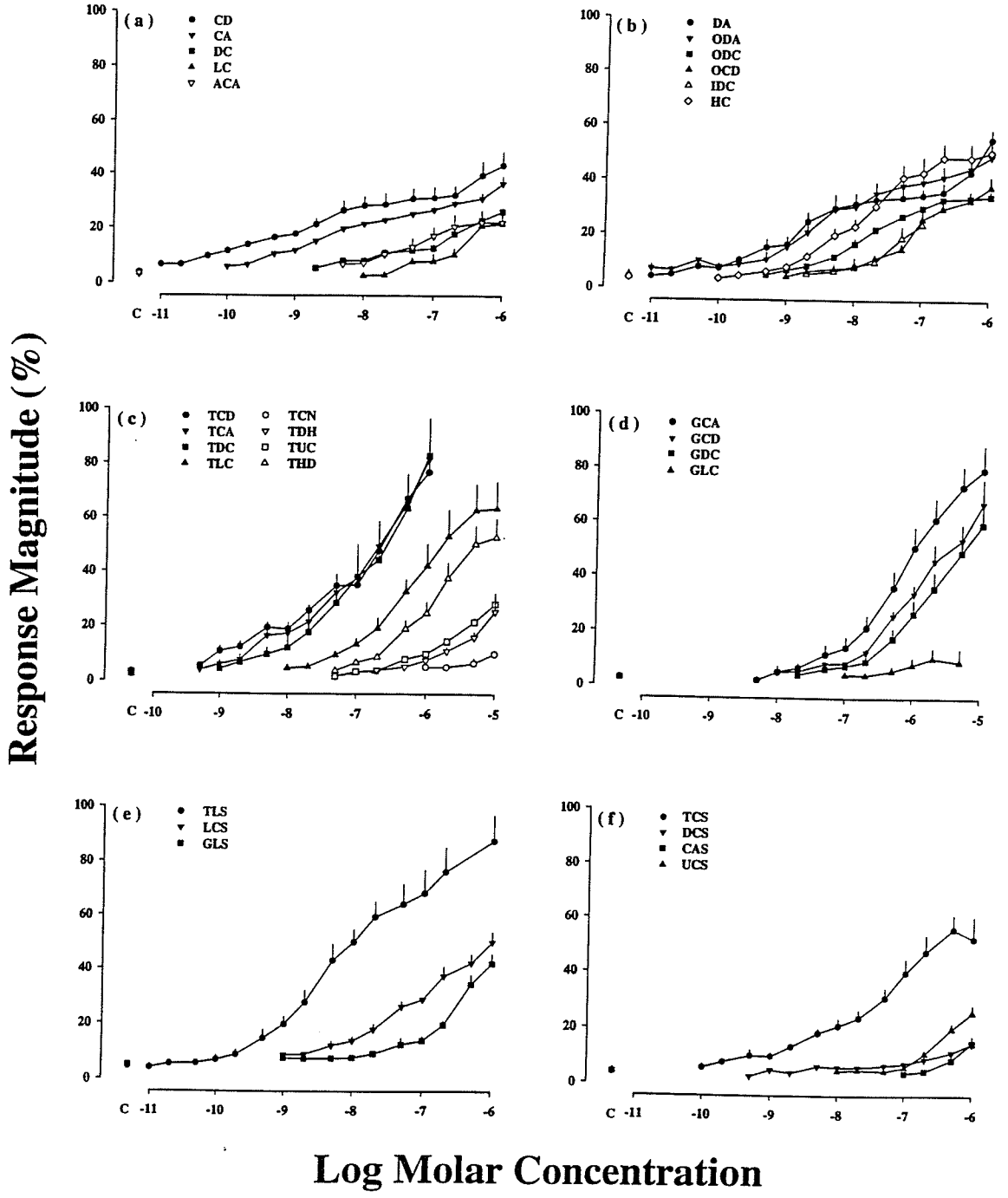


Figure 2.4 Concentration-response (C-R) relationships of phasic responses for bile acids. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. **(a)** and **(b)**, free bile acids; **(c)** and **(d)**, amidated bile acids; **(e)** and **(f)**, sulphated bile acids. C=control response. Note the different X-axis scales. Refer to Table 2.1 for abbreviations.

3-hydroxyl made little difference on stimulatory effectiveness (DC *vs* IDC). By contrast, 5α- configuration bile acids was much less stimulatory than the 5β-counterparts (ACA *vs* CA). Furthermore, an extra hydroxyl at position C_6 decreased the stimulant potency (HC *vs* CD). Shortening the five-carbon side chain reduced the stimulatory effectiveness by 100 times (NDC *vs* DC). A cholestane, 5β-epicholestanol (CE), with more carbon atoms in the side chain and with no charged carboxylic group at the terminal, was nonstimulatory (Table 2.1).

For all amidated nonsulphated bile acids tested, response magnitudes increased nearly exponentially with logarithmic increases in concentrations. The C-R curves for taurine and glycine amidated bile acids were similar, except that the C-R curves for the latter shifted parallel to the right (Fig. 2.4c, d).

Of amidated bile acids, TCD and TCA were the most stimulatory. It appeared that an hydroxyl at C_3, C_7 or C_{12} was necessary for stimulatory effectiveness. Taurocholanic acid (TCN), lacking hydroxyls, and taurodehydrocholic acid (TDH), having oxo substituents only, were ineffective in inducing olfactory responses. Taurodeoxycholic acid was less stimulatory than TCD, indicating 7α-hydroxyl is more influential for effective stimulation than 12α-hydroxyl. However, β orientation of 7-hydroxyl diminished the stimulant activity (TUC *vs* TCD). The hydroxyls at other positions reduced the potency of stimulants (TLC *vs* THD). In glycine amidated bile acids, GCA was more stimulatory than GCD, in contrast to similar stimulatory power between TCA and TCD (Fig. 2.4c, d; Table 2.1).

Taurolithocholic acid 3-sulphate was also one of the most potent bile acids

tested, with threshold of 0.02 nM. Its C-R curve was distinct from those for free or amidated nonsulphated bile acids in the low threshold and greater response magnitudes at fairly low concentrations. The shapes of C-R curves of other sulphated bile acids were more similar to that of TLS than to those of free or amidated bile acids (Fig.2.4e, f).

Among sulphated bile acids, molecules with both taurine amidation at C_{24} and sulphation on 3α-hydroxyl were the most stimulatory (e.g. TLS and TCS). Glycolithocholic acid 3-sulphate (GLS) was much less effective for olfactory responses than its equivalent free or taurine amidated forms. An additional sulphation on C_7 or C_{12} hydroxyl was not required (TCS *vs* TLS) and might reduce the molecule's affinity to receptors (DCS and CAS *vs* LCS).

Olfactory specificity for bile acids

Self-adaptation

Self-adaptation is a special case of cross-adaptation in which the adapting and test compounds are the same. Self-adaptation was conducted before cross-adaptation to examine the behaviour of interactions between bile acids and the prospective receptors. When the olfactory epithelium was adapted to 10^{-8} M or 10^{-7} M TCA, C-R curves for TCA shifted along the concentration axis over adaptation ranges, but the shape stayed consistent (Fig. 2.5). Similar parallel shift patterns of C-R curves were obtained when olfactory responses to TLS and CD were tested under self-adaptation conditions using adaptation concentrations of 10^{-9} and 10^{-8} M. The K_B remained

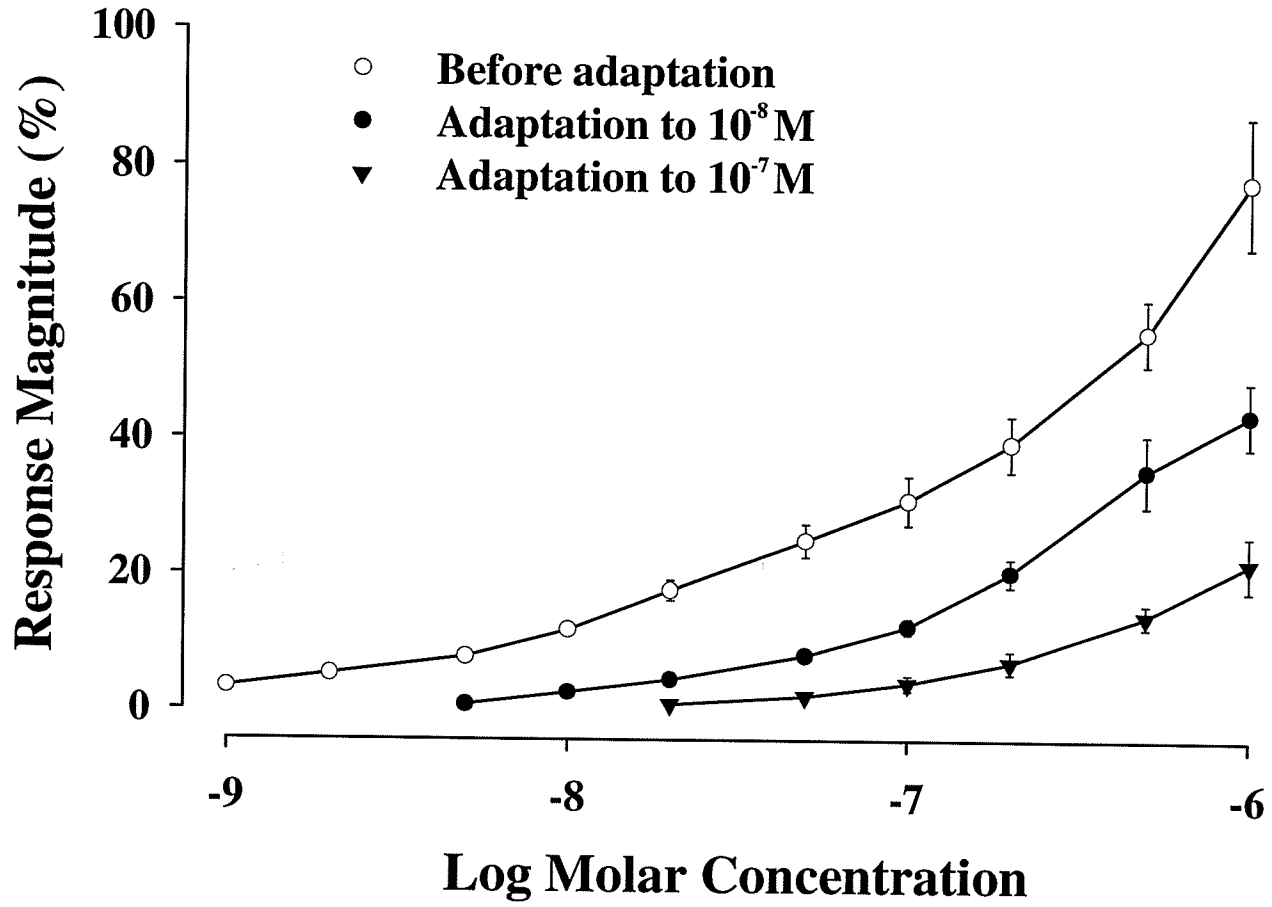


Figure 2.5 Concentration-response (C-R) relationships of taurocholic acid (TCA) before (○) and during self-adaptation at 10^{-8} M (•) and 10^{-7} M (▾), an example of parallel shifts of concentration-response (C-R) curves during self-adaptation in three representative bile acids: TCA, chenodeoxycholic acid (CD) and taurolithocholic acid 3-sulphate (TLS). Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine.

constant during self-adaptation for each of the three chemicals.

Receptor specificity for bile acids

Bile acids tested above can be tentatively categorized into at least three groups based on C-R relationships: free bile acids (Fig. 2.4a, b), amidated bile acids (Fig. 2.4c, d) and sulphated bile acids (Fig. 2.4e. f). Cross-adaptation may reveal whether multiple patterns of C-R relationships for bile acids are mediated by separate receptors. On the basis of the olfactory sensitivity and C-R relationship data obtained previously, potent bile acids representing each bile acid group were used as major adapting compounds. Near saturation concentrations were used during adaptation to ensure expected receptors were properly saturated.

Adaptation to free bile acids: The C-R series of seven free, three taurine amidated, one glycine amidated and two sulphated bile acids were examined before and during adaptation to 10^{-7} M CD. The thresholds of olfactory responses to amidated and sulphated bile acids were not affected by adaptation. Moreover, responses to amidated bile acids were only reduced at high concentrations during adaptation (Fig 2.6). By contrast, olfactory responses to all free bile acids were totally eliminated up to 10^{-7} M during adaptation, except for DA (Fig. 2.7a). The latter is a more potent free bile acid than CD (Fig. 2.4, Table 2.1). It elicited $13.0 \pm 3.2\%$ of original responses at 10^{-7} M, but none at concentrations below 10^{-7} M during adaptation. When 10^{-7} M CA was the adapting compound, the adaptation effect was similar to that of CD; responses to the free bile acids were completely inhibited,

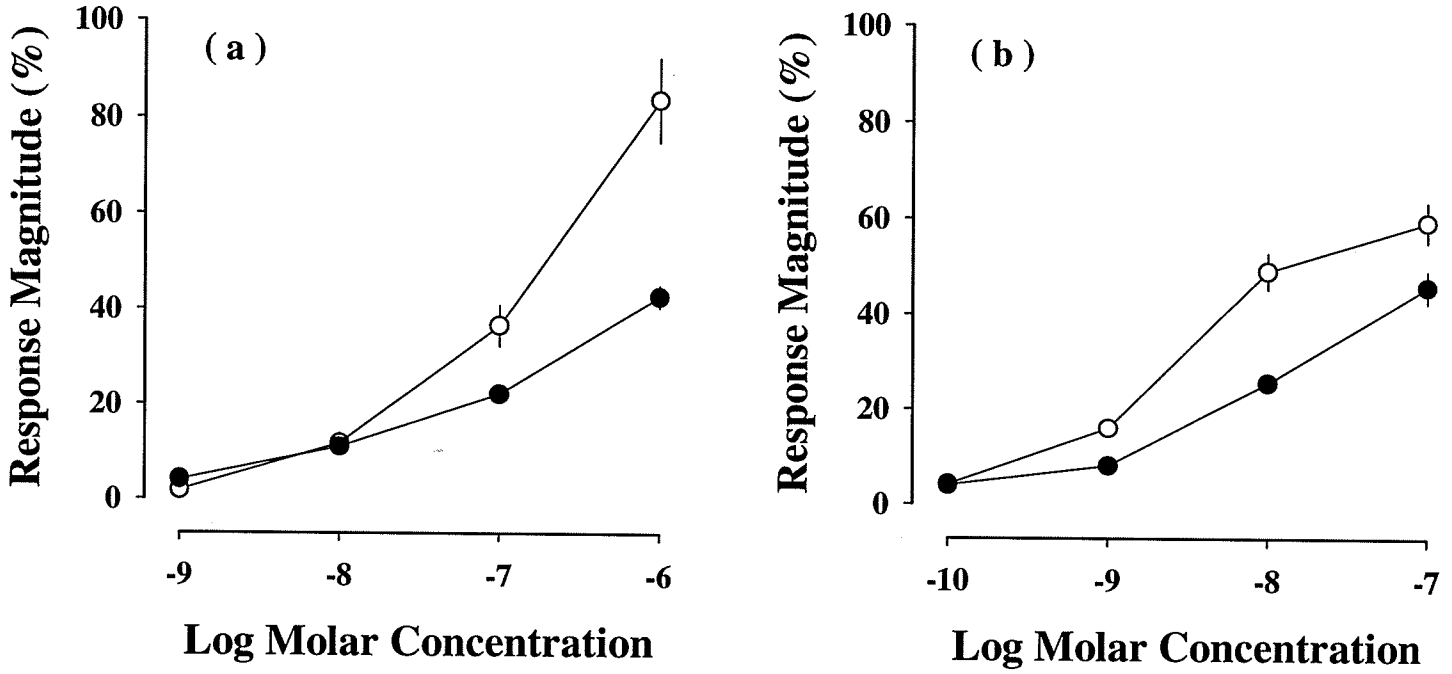


Figure 2.6 Concentration-response (C-R) relationships of **(a)** taurocholic acid (TCA) and **(b)** taurolithocholic acid 3-sulphate (TLS) before (○) and during (•) adaptation to 10^{-7} M chenodeoxycholic acid (CD). Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine.

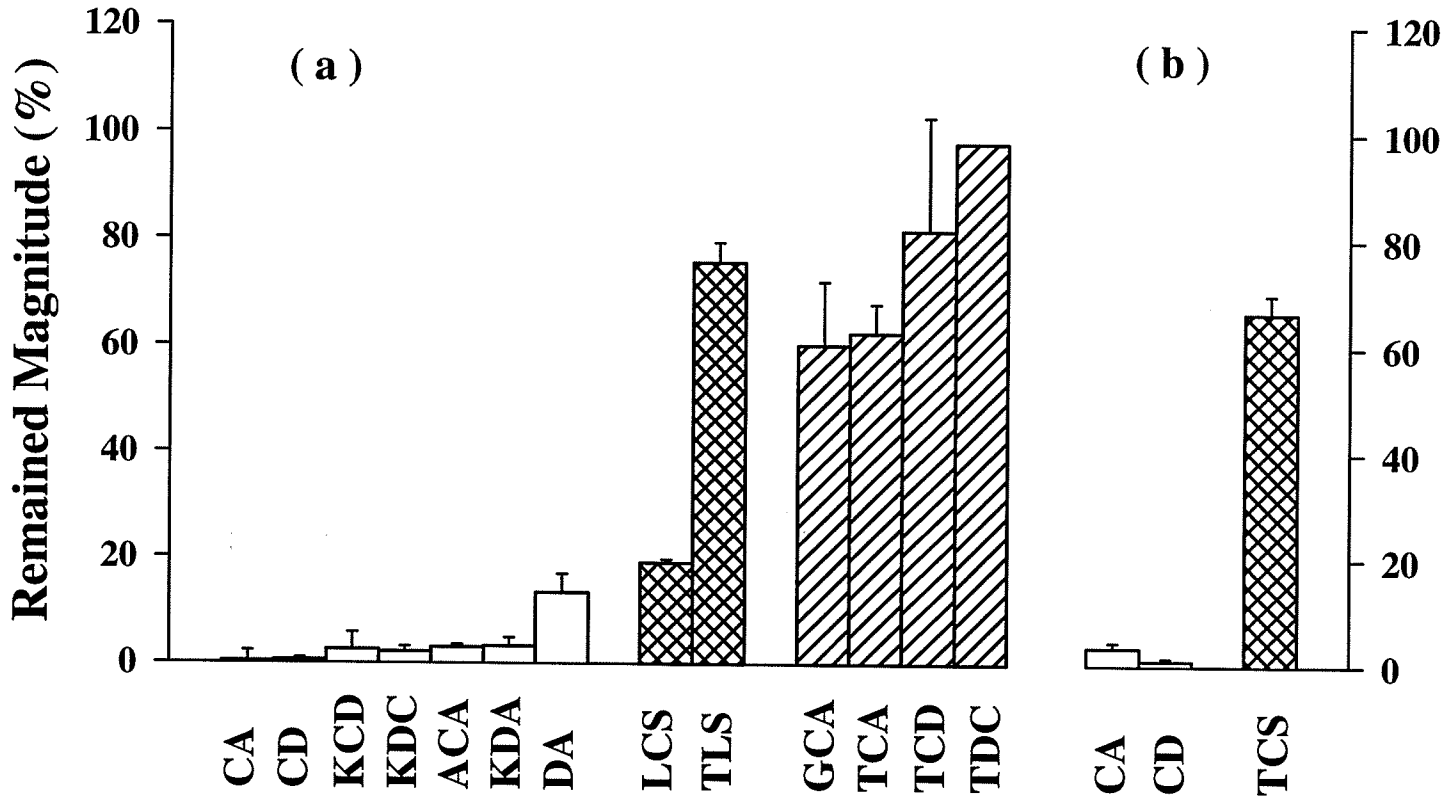


Figure 2.7 Remained response magnitudes at 10^{-7} M, plotted as percentages of equivalent original responses, during adaptation to **(a)** 10^{-7} M chenodeoxycholic acid (CD) and **(b)** 10^{-7} M cholic acid (CA). Test bile acids are grouped into free bile acids (open bars), sulphated bile acids (crosshatched bars) and amidated bile acids (oblique bars). Refer to Table 2.1 for abbreviations.

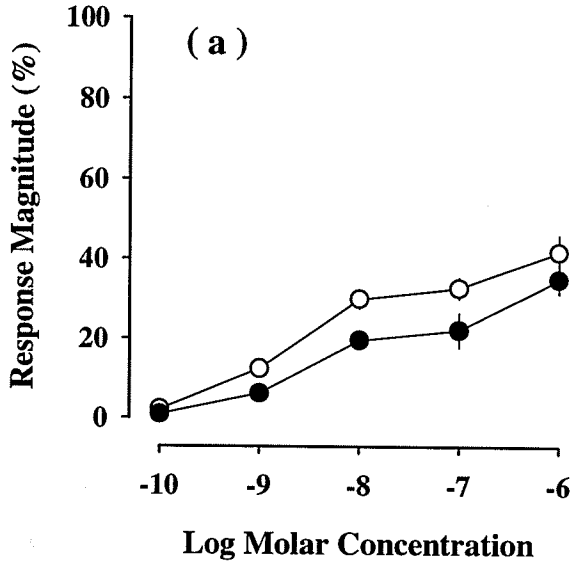
while the response to a sulphated bile acid was partially reduced (Fig 2.7b).

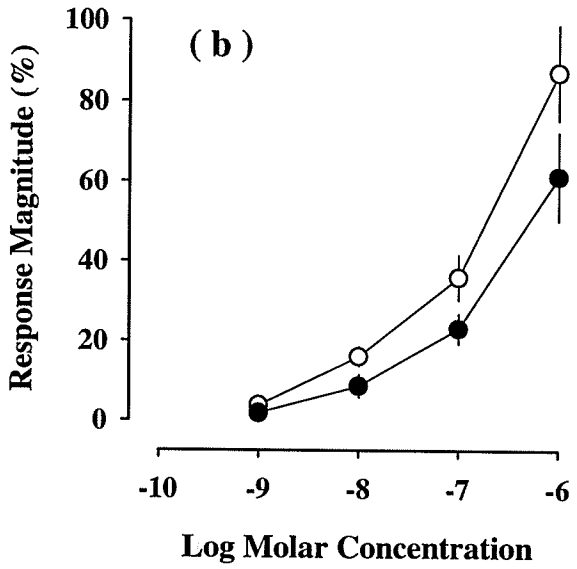
Adaptation to sulphated bile acids: Olfactory responses to GLS and TCS were completely inhibited even when stimulated at 10^{-6} M during adaptation to 10^{-7} M TLS. When adapted to either 10^{-7} M TLS or 10^{-6} M TCS, olfactory responses to TCA or CD were partially inhibited throughout concentration series (Fig. 2.8).

Olfactory responses to TLS were completely inhibited up to 10^{-8} M during adaptation to 10^{-6} M TCS (Fig. 2.8). This was a maximum reduction by a single adapting compound among TCS, CD, TCA, and GCA. When adapted to a mixture of 10^{-6} M TCS and 10^{-6} M TCA, olfactory responses to 10^{-7} M TLS were abolished. In contrast, 39% of the original response to 10^{-7} M TLS remained during adaptation to a trinary mixture, 10^{-7} M CD, 10^{-6} M TCA and 10^{-4} M L-cysteine (CYS).

Adaptation to amidated bile acids: Olfactory responses to all taurine and glycine amidated bile acids were completely inhibited during adaptation to 10^{-6} M TCA. However, 15.1 ± 2.17% of the original response to CA and 25.1 ± 3.78% to TLS remained at the test concentration as low as 10^{-8} M during adaptation. Figure 2.9 shows the remaining olfactory response magnitudes for five bile acids at 10^{-7} M, as compared to the original response, during adaptation to 10^{-6} M TCA.

Olfactory responses to 10^{-8} M TCA were maximally inhibited during adaptation to 10^{-6} M GCA, as compared to adaptation to CD, TCS or TLS (Fig. 2.6; 2.8; 2.10). This inhibition was even stronger than the effect of a 10^{-6} M CD and 10^{-7} M TLS mixture adaptation. The latter reduced TCA responses to 47.7 ± 1.4% of the original level ($P < 0.01$). Reductions of olfactory responses to TLS during adaptation to





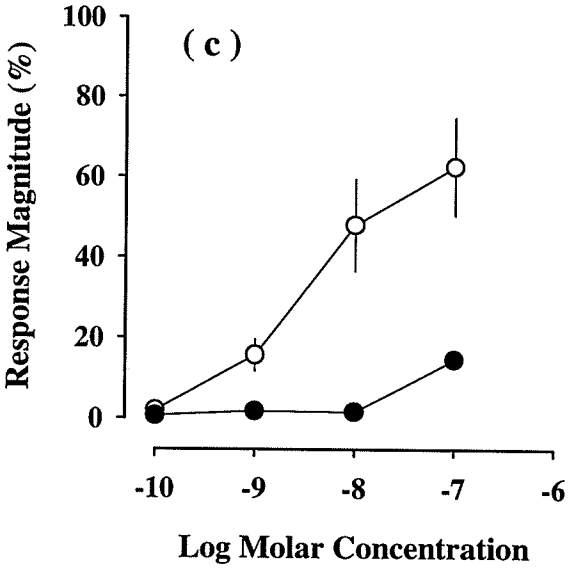


Figure 2.8 Concentration-response (C-R) relationships of **(a)** chenodeoxycholic acid (CD), **(b)** taurocholic acid (TCA) and **(c)** taurolithocholic acid 3-sulphate (TLS) before (○) and during (•) adaptation to 10^{-6} M taurocholic acid 3,7,12-trisulphate (TCS). Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. The adapting effect of 10^{-7} M TLS on olfactory responses to CD and TCA was essentially the same as shown.

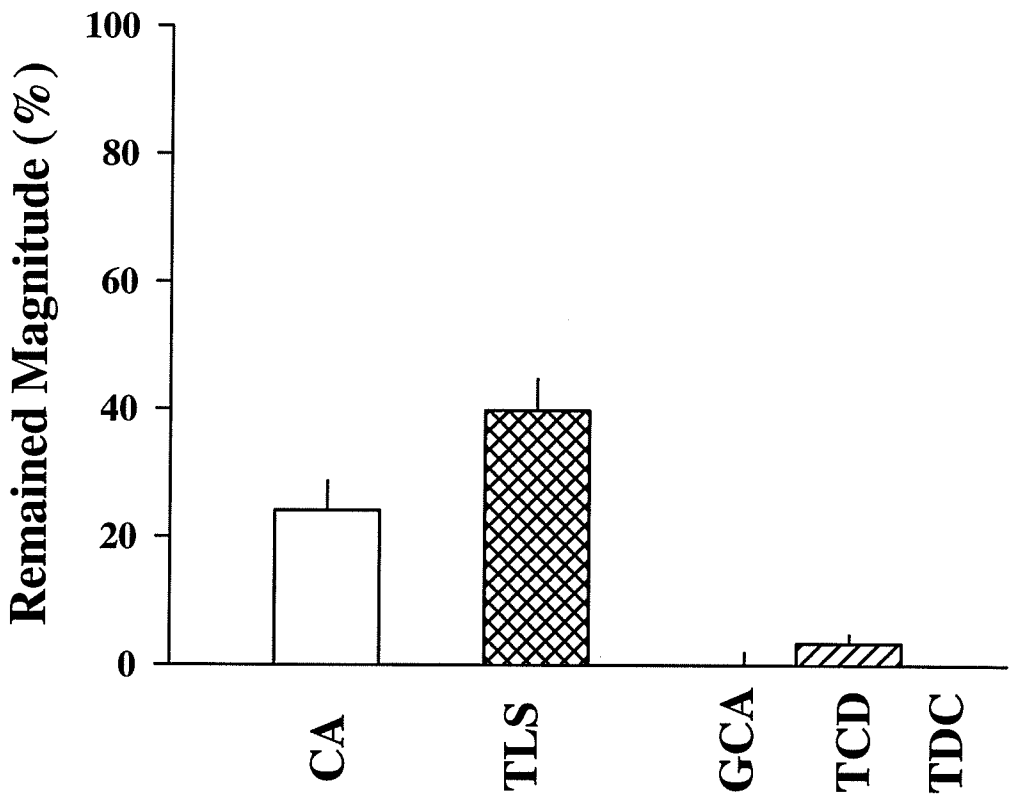


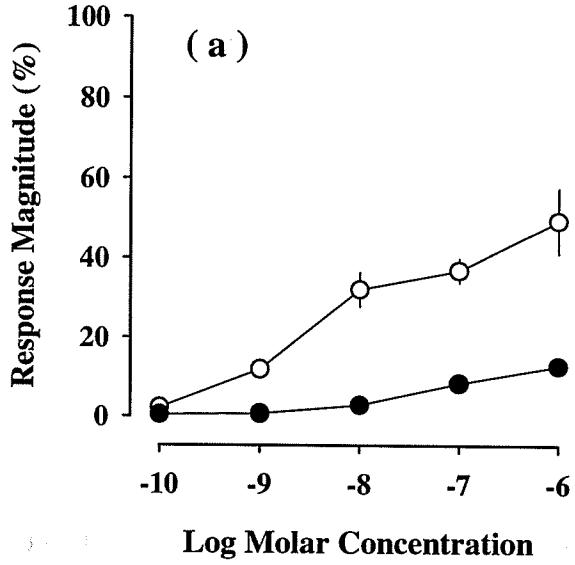
Figure 2.9 Remained response magnitudes at 10^{-7} M, plotted as percentages of equivalent original responses during adaptation to 10^{-6} M taurocholic acid (TCA). Bar filling patterns are the same as Figure 2.7. CA=cholic acid; TLS=taurolithocholic acid 3-sulphate; GCA=glycocholic acid; TCD=taurochenodeoxycholic acid; TDC=taurodeoxycholic acid.

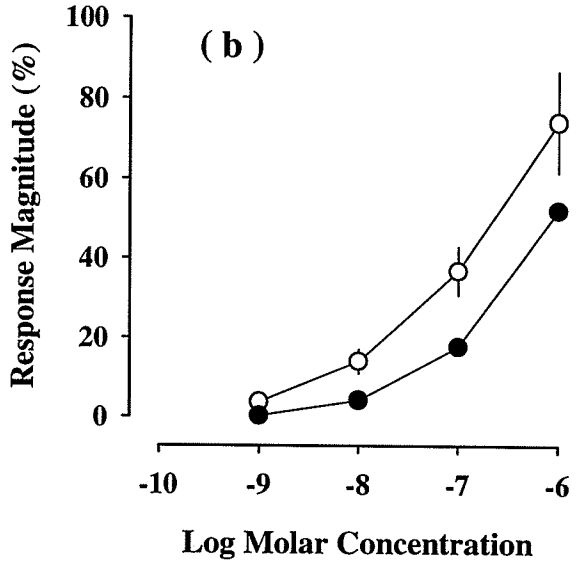
either GCA or TCA were similar. However, it appeared that GCA was a better inhibitor for free bile acids, as opposed to TCA (Fig. 2.10). About 90% of olfactory responses of 10^{-8} M CD was inhibited during adaptation to GCA.

Receptors for PZS: Petromyzonol sulphate (3α,7α,12α,24-tetrahydroxy-5α-cholan-24-sulphate) is a bile acid produced by sea lamprey. Presence of an independent receptor for PZS has been suggested in this species (Li et al. 1995). Petromyzonol sulphate was also a potent olfactory stimulant for lake char, with threshold of 1 nM (Table 2.1). Thus, a single to four-compound mixture adaptation was applied to examine relationships between PZS and other bile acids. Figure 2.11 illustrates olfactory responses to PZS at 10^{-7} M before and during adaptation to various compounds. Olfactory responses to PZS were reduced to approximately half of the original during adaptation to either 10^{-6} M CD, TLS or TCA. Responses to PZS were reduced to one-third to one-fourth the original during adaptation to a mixture of 10^{-6} M CD and TLS, or a mixture of 10^{-6} M TLS and TCA. Olfactory responses to PZS were still detectable when adapted by a mixture of 10^{-6} M CD, TCA and TLS, or by a mixture of 10^{-6} M CD, TLS and TCA, and 10^{-5} M CYS.

Binary mixture experiments

To strengthen the results obtained in the previous sections, I further conducted binary mixture experiments between bile acids to see if there are distinct receptors for each group of bile acid. The response to a binary mixture stimulation is expected equivalent to the sum of those to individual components in the mixture, if two stimuli





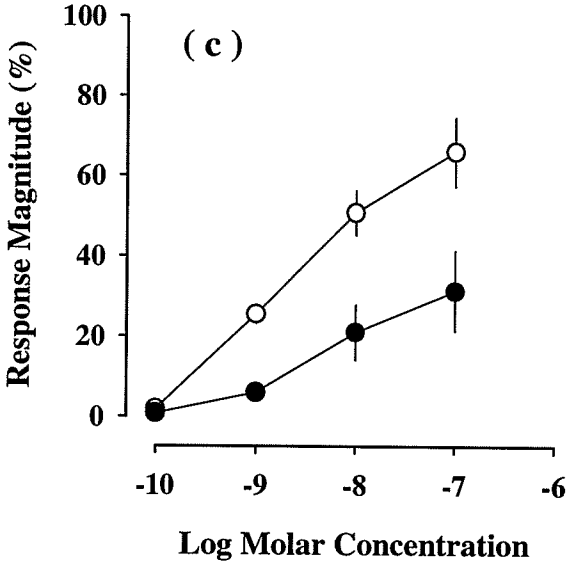


Figure 2.10 Concentration-response (C-R) relationships of **(a)** chenodeoxycholic acid (CD), **(b)** taurocholic acid (TCA) and **(c)** taurolithocholic acid 3-sulphate (TLS) before (○) and during (•) adaptation to 10^{-6} M glycocholic acid (GCA). Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine.

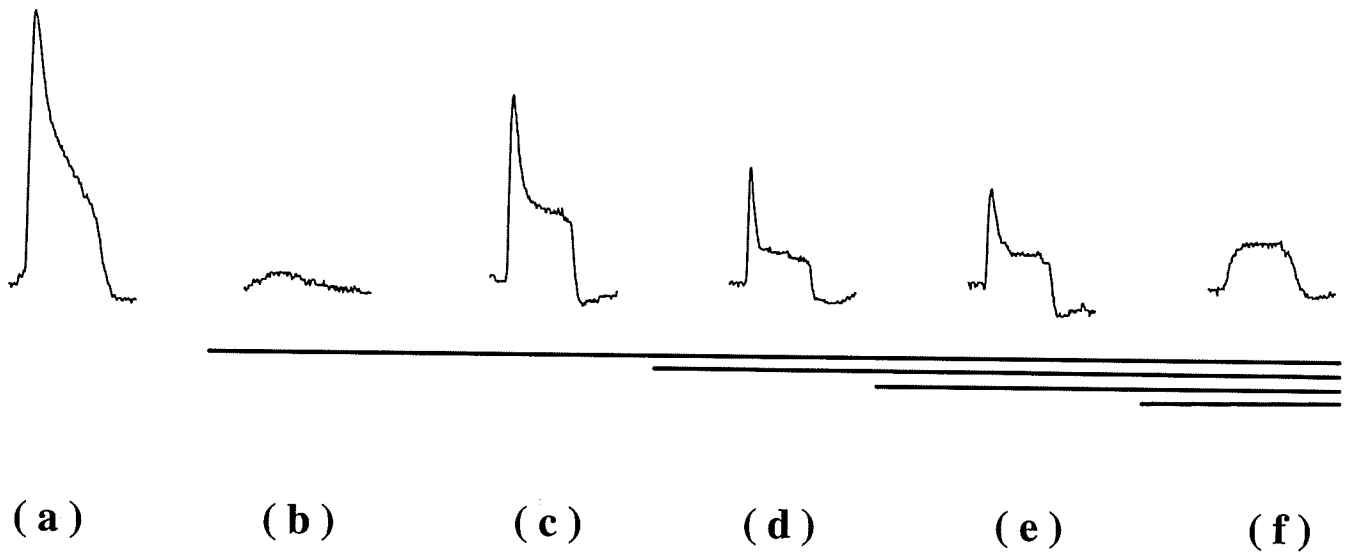


Figure 2.11 Electroolfactogram (EOG) responses to petromyzonol sulphate at 10^{-7} M before and during adaptation to a single to four-compound mixtures. **(a)** response before adaptation; **(b)** control response; **(c)** during adaptation to 10^{-6} M chenodeoxycholic acid (CD). The reduction of the responsiveness to PZS due to 10^{-6} M taurocholic acid (TCA) or 10^{-6} M taurolithocholic acid 3-sulphate (TLS) adaptation is similar as shown; **(d)** during adaptation to a mixture of 10^{-6} M CD and TLS. Responses to PZS during adaptation to a mixture of 10^{-6} M TCA and TLS is slightly smaller than shown; **(e)** during adaptation to a mixture of CD, TLS and TCA at 10^{-6} M; **(f)** during adaptation to a mixture of 10^{-6} M CD, TLS, TCA and 10^{-5} M L-cysteine. The lines underneath indicate numbers of adapting compounds.

interact with different receptors and their chemical natures are independent in the mixture. Figure 2.12 illustrates responses to binary mixtures of bile acids across groups and their relations with those to individual components induced separately. Responses to a mixture of TCA and CD, TLS and CD, or TCA and TLS were equal to the sum of responses to individual components at the resulting concentrations in the mixtures. Olfactory responses to mixture stimulation were further compared with individual components at equal molarity. Responses to a binary mixture were equal or greater than the responses to either of individual components at equal molarity. This suggests that olfactory responses to the mixture stimulation are not simply due to the increased molarity in the mixture solution. Response suppression or synergism to binary mixtures was not observed (paired t-test $P > 0.6$).

Relationships with other olfactory stimuli

Figure 2.13 shows C-R relationships of CD, PGF, CYS and 17,20P. They respectively represent four classes of olfactory stimuli for fish, bile acids, PGs, amino acids and gonadal steroids, (ref. Chapter 1). Besides CYS, PGF was a potent olfactory stimulus for lake char. The threshold for PGF was 0.001 nM and responses saturated around 10^{-9} M. However, 17,20P was nonstimulatory.

To determine whether these stimulants interact with bile acid receptors, I examined olfactory responses to CD, TCA and TLS, three representative bile acids, during adaptation to either 10^{-3} M CYS, 10^{-3} M L-serine (SER), or $2x10^{-9}$ M PGF. Olfactory responses to the bile acids were not affected by either of these adaptations

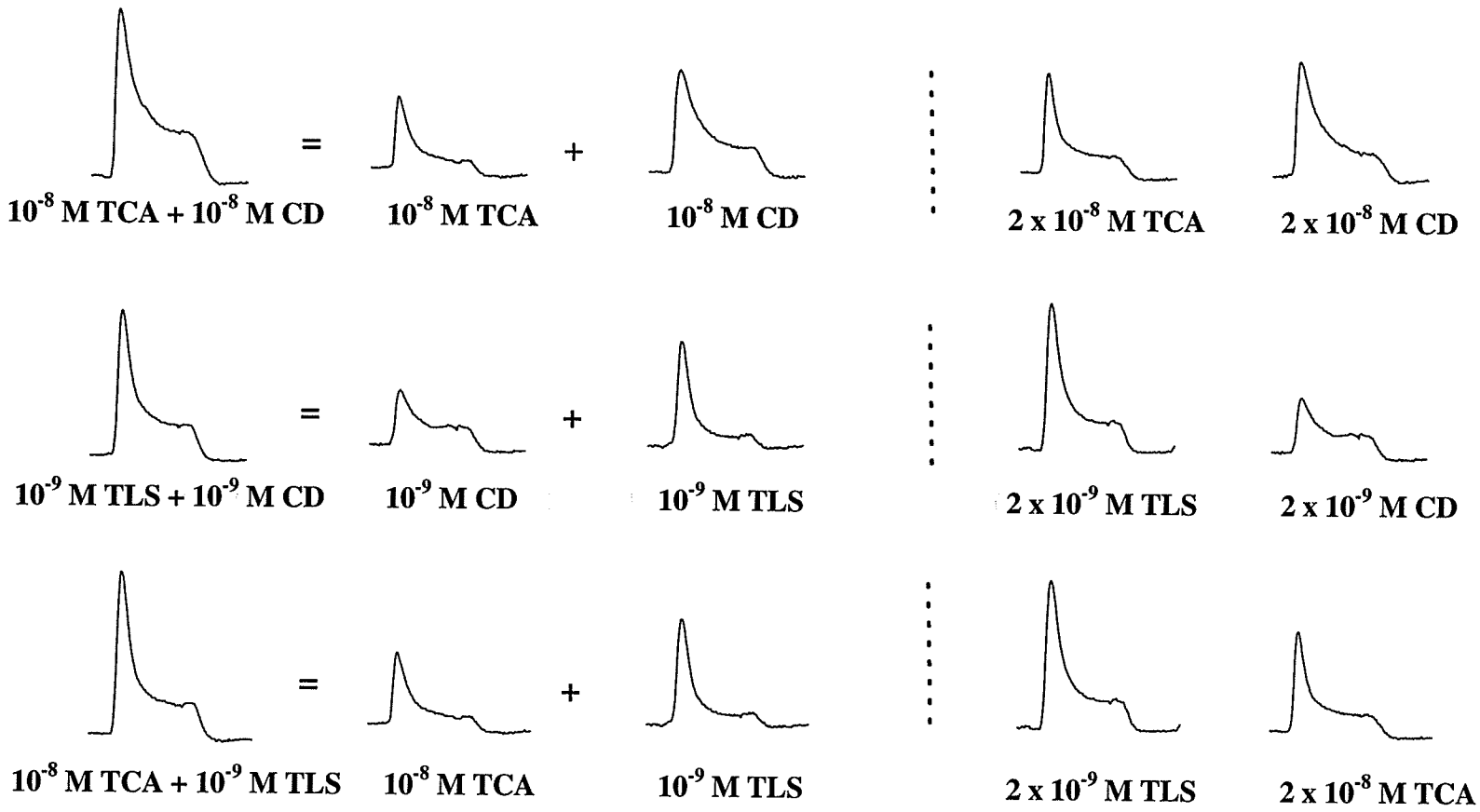


Figure 2.12 Electroolfactogram(EOG) responses to binary mixtures are compared with responses induced by individual components at the resulting concentration in the mixture. Olfactory responses to doubled concentrations of individual components in the mixture are shown on the right for comparison.

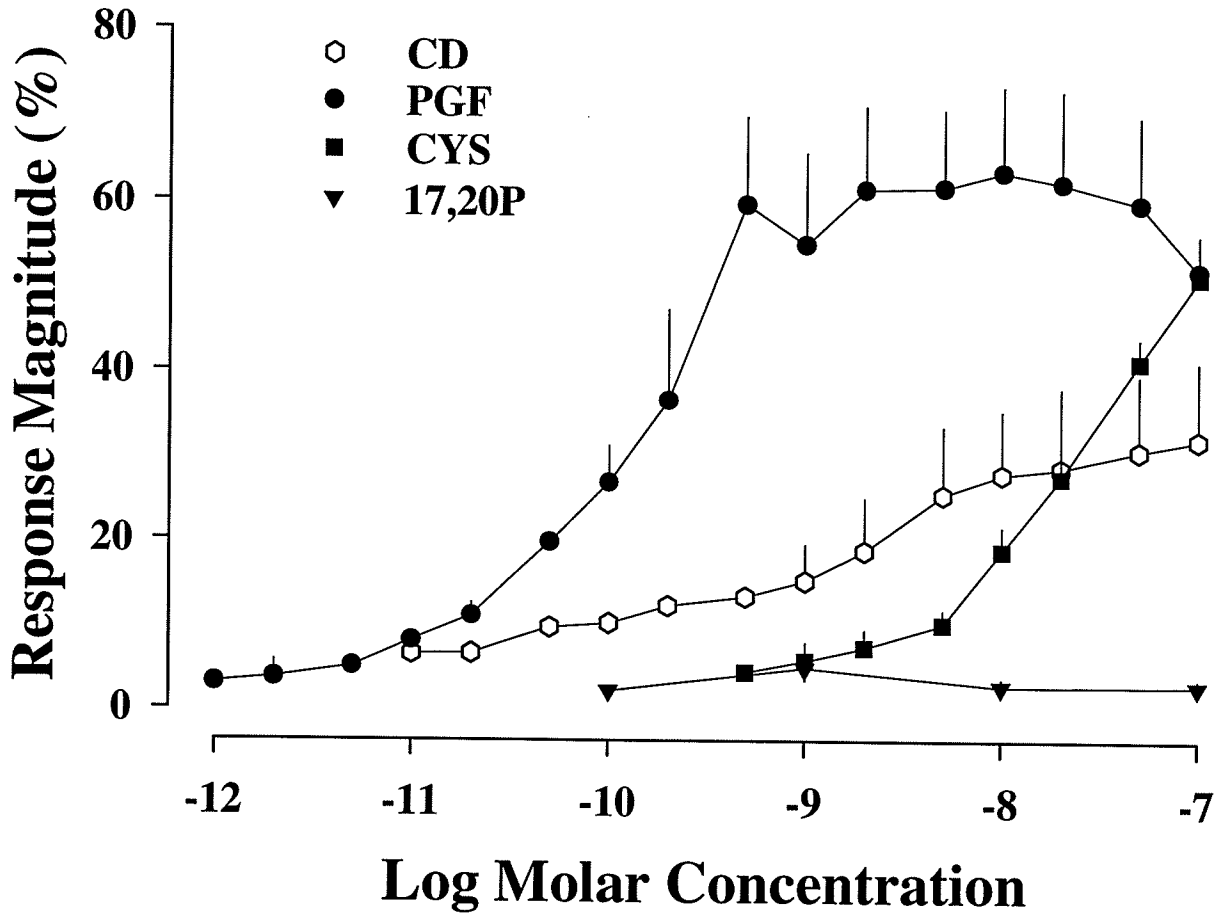


Figure 2.13 Concentration-response (C-R) relationships of four chemicals representing four major classes of fish olfactory stimuli, bile acids, prostaglandins, amino acids and gonadal steroids. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. CD=chenodeoxycholic acid; PGF=prostaglandin $F_{2\alpha}$; CYS=L-cysteine; 17,20P=17α,20β-dihydroxy-4-pregnen-3-one.

(Fig.2.14).

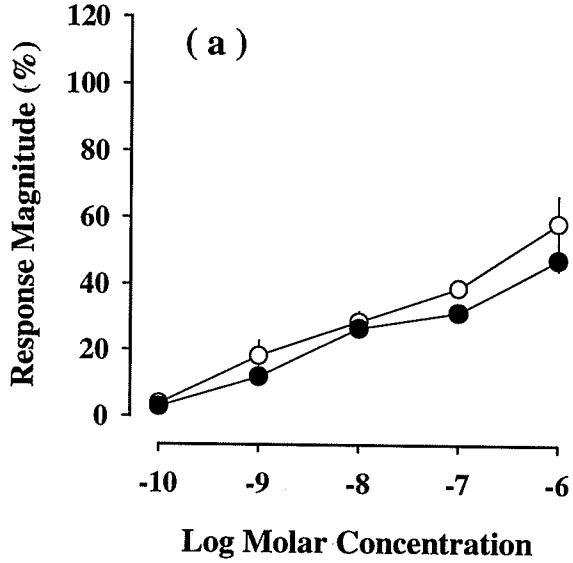
Reciprocal cross-adaptation further revealed that C-R relationships of CYS and ARG, two representative amino acids (Sveinsson and Hara 1990b), were unchanged before and during adaptation to either 10^{-7} M CD, 10^{-6} M TCA or 10^{-7} M TLS (Fig.2.15). In addition, responses to 10^{-7} M $PGF_{2\alpha}$ were not affected by adaptation to either 10^{-7} M TLS, or a mixture of 10^{-7} M CD and 10^{-6} M TCA (paired t-test $P > 0.3$).

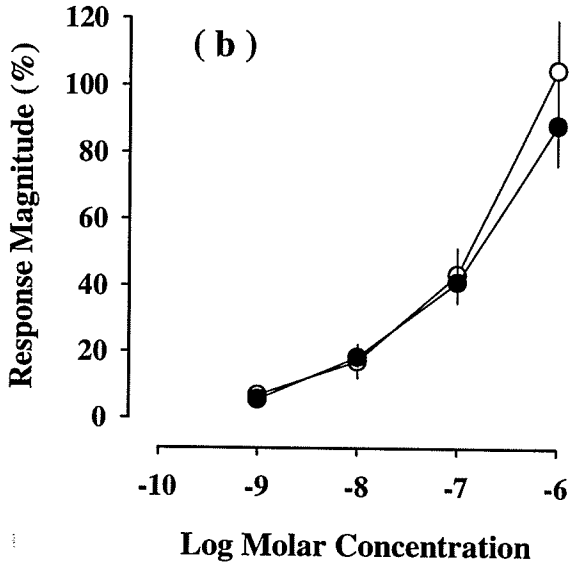
Olfactory responses to 10^{-9}-10^{-7} M CD, TCA or TLS were unchanged with or without the presence of 10^{-6} M 17,20P in the stimulating solutions.

Olfactory sensitivity to bile acids at different life stages

Olfactory responses

Lake char embryos incubated under the conditions described (Materials and Methods) took more than 80 days before sporadic hatching was observed. Examination of olfactory responses in lake char embryos started at stage $E^{3}6$ when shallow olfactory placodes were visible from a lateral or ventral view of the head. Extracellular spike activities in response to TCA were obtained at the beginning of stage $E^{4}7$. The concentration-dependent response pattern became evident in stage $F^{1}9$ eleutheroembryos (Fig. 2.16). This was associated with an increasing number of sensory neurons (see below). However, SER at 10^{-6}-10^{-4} M did not elicit any responses in all embryos and eleutheroembryos examined at any regions on the placode. Even an amino acid mixture, which triggers responses 4-6 times larger than





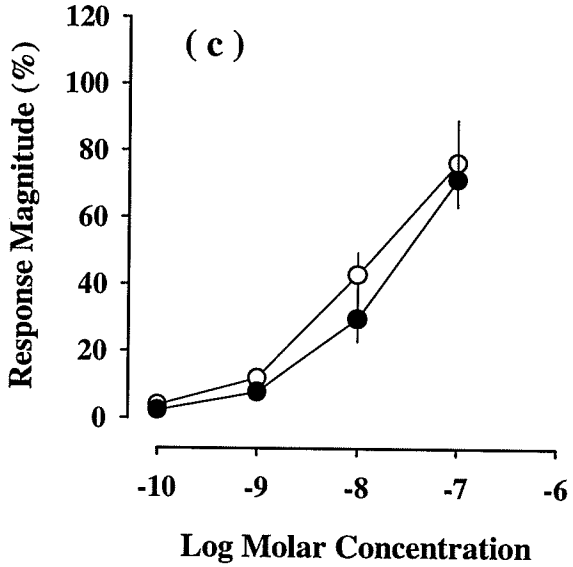


Figure 2.14 Concentration-response (C-R) relationships of three representative bile acids **(a)** chenodeoxycholic acid (CD), **(b)** taurocholic acid (TCA), and **(c)** taurolithocholic acid 3-sulphate (TLS) before (○) and (•) during adaptation to $2x10^{-9}$ M prostaglandin $F_{2\alpha}$(PGF). Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. C-R curves of the above three bile acids before and during adaptation to 10^{-3} M L-cysteine (CYS) and 10^{-3} M L-serine (SER) are essentially the same as shown.

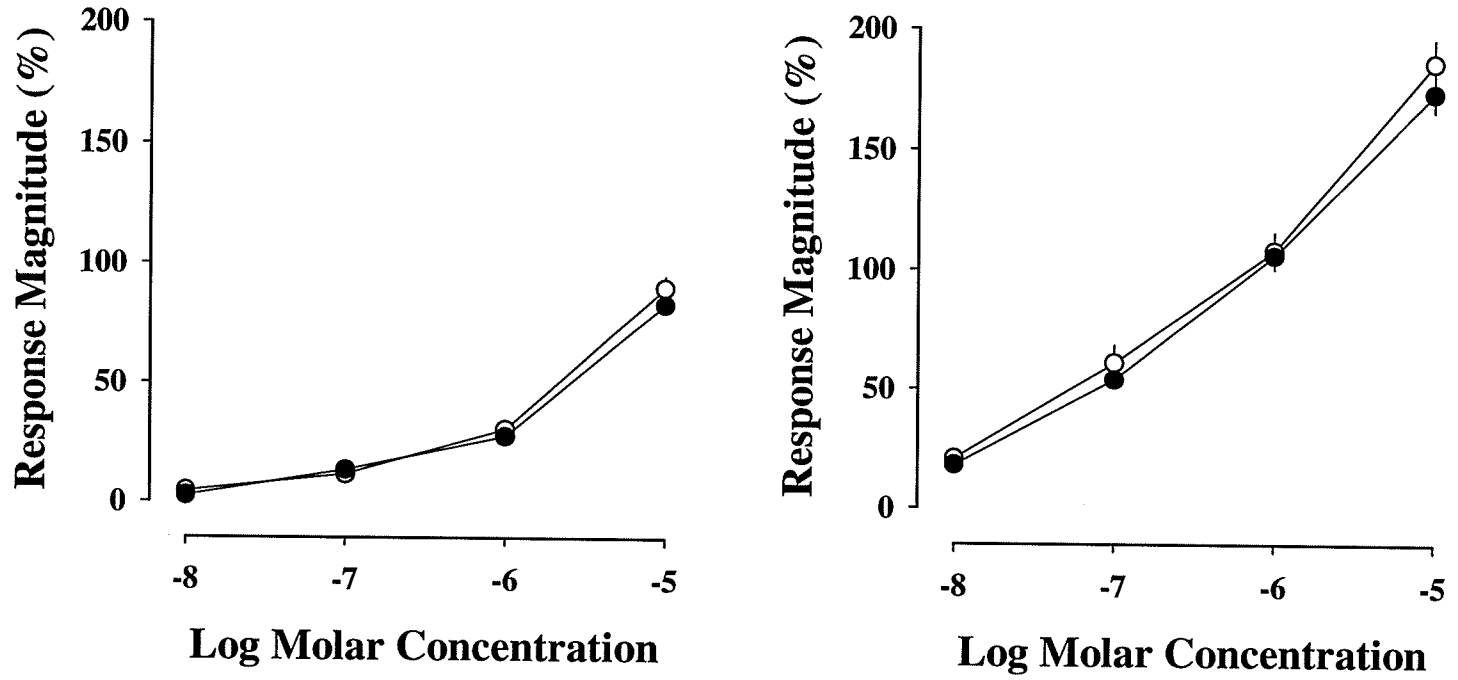


Figure 2.15 Concentration-response (C-R) relationships of L-cysteine (CYS) **(a)** and L-arginine (ARG) **(b)** before (○) and (•) during adaptation to 10^{-6} M taurocholic acid. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. C-R curves of CYS and ARG before and during adaptation to 10^{-7} M chenodeoxycholic acid (CD) or 10^{-7} M taurolithocholic acid 3-sulphate (TLS) are essentially the same as shown.

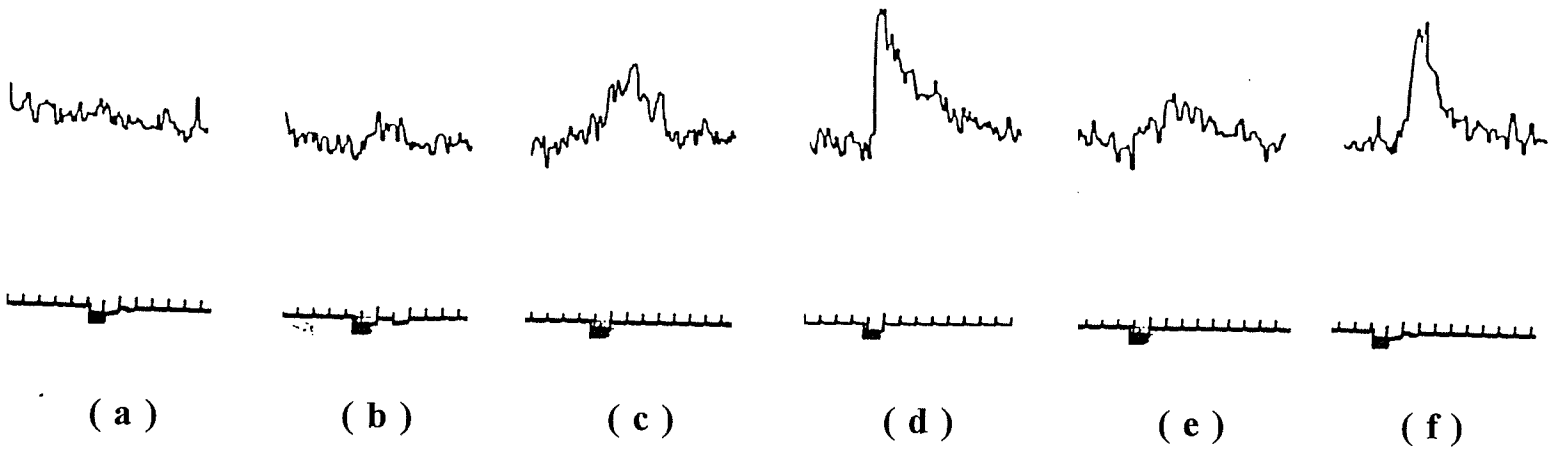


Figure 2.16 Olfactory neural responses of stage $F^{1}9$ lake char eleutheroembryos to **(a)** 10^{-5} M L-serine (SER), **(b)** amino acid mixtures, **(c)** $5x10^{-7}$ M taurocholic acid (TCA), **(d)** 10^{-6} M TCA, **(e)** 10^{-7} M taurolithocholic acid 3-sulphate (TLS), and **(f)** 10^{-6} M TLS.

the STD in juveniles, failed to induce a response.

Olfactory responses to 10^{-5} M SER were detected in stage $A^1$11 alevins (total length around 30 mm). The relative response magnitudes for TCA were identical throughout the concentration series when tested in 0^+ age (total length less than 10 cm) and in 2-4 yr old lake char (Fig. 2.17).

A total of 6 reproductively mature lake char (3 spawning males, 1 prespawning female, 1 day-post-spawning female and 1 month-before-spawning female) were tested for EOG responses to bile acids. The detection thresholds for four bile acids, CD, TCA, TLS and PZS were similar between the adults and juveniles. Statistical analysis suggested that relative response magnitudes obtained in these mature fish did not differ from those in juveniles ($P > 0.05$).

Olfactory sensory neurons

Whether fish olfactory responses to bile acids and amino acids are mediated by two morphologically distinct olfactory sensory neurons, ciliated sensory neuron (CSN) and microvillar sensory neuron (MSN) is one of the most interesting topics in fish chemoreception. Temporal differentiation of olfactory responses to bile acids and amino acids at early developmental stages of lake char has provided an opportunity to examine relationships between the types of sensory neurons and olfactory responses to stimuli.

Scanning electron microscopy revealed that the olfactory placode surface had only regularly spaced low microvilli at the early-mid $E^3$6 stage. A young ciliated

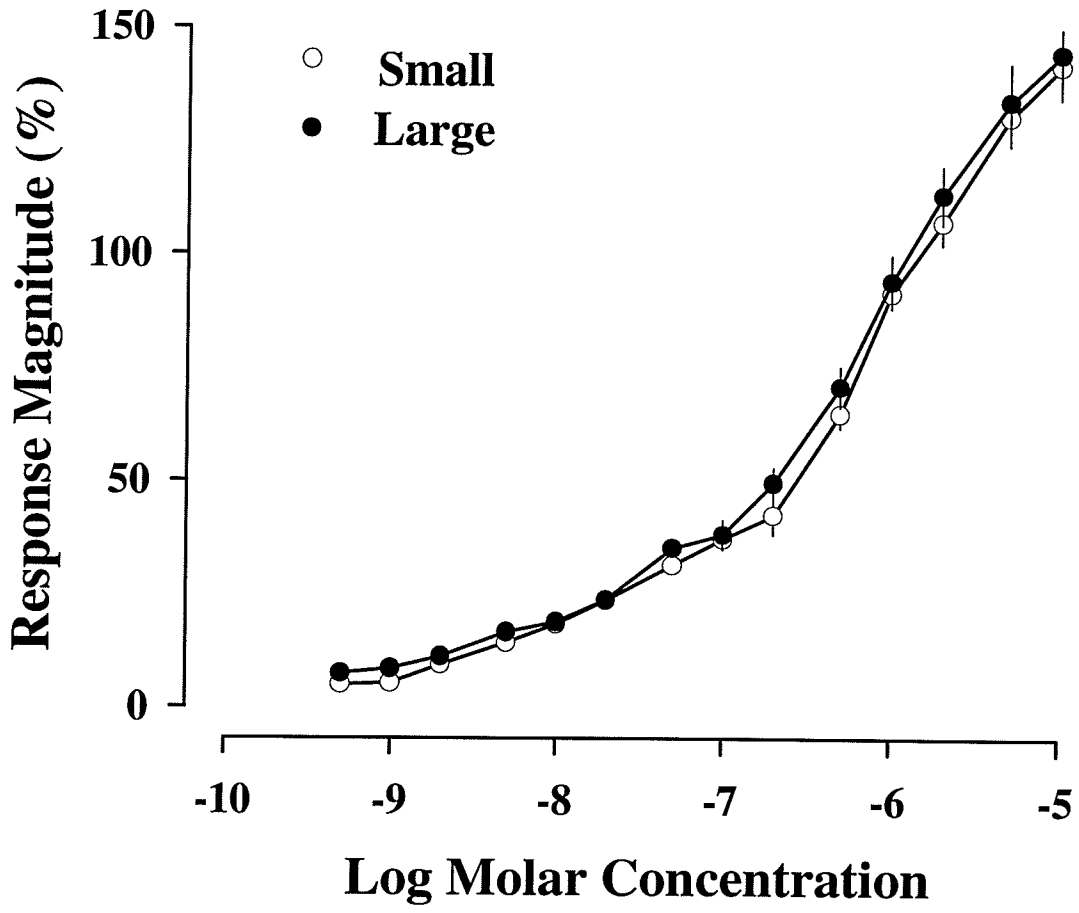


Figure 2.17 Concentration-response (C-R) curves of taurocholic acid (TCA) examined in the lake char of different ages. (○) from 0^+ age juveniles of total length less than 10 cm; (•) from 3 yrs old juveniles. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine.

sensory neuron, recognized by its swollen apical knob with two short cilia extending at lateral angles from the knob, was first seen in the olfactory placode at the mid $E^{3}6$ stage (Fig. 2.18a). The number of CSNs increased as the embryo development progressed. At the $E^{5}8$ stage, CSNs predominated in olfactory placodes. Most CSNs had more cilia than before. Meanwhile, 2-3 young MSNs were also observed (Fig. 2.18b). Figure 2.18c is a scanning electron micrograph of surface of a posterior lamella in juvenile lake char (TL=20 cm), showing the morphology of mature CSNs and MSNs.

DISCUSSION

General characteristics of EOG responses

The EOG method has been widely used in chemoreception studies in vertebrates because of its minimal surgery requirement, technical simplicity and stability during recording. Early studies suggested that the EOG is the summated generator potential evoked through extracelluar resistance of the olfactory epithelium (Ottoson 1956, 1971, 1974; Getchell 1974). Evidence from recent whole-cell recording has lent support to this notion (Lowe and Gold 1991). However, the nature of EOG features remains controversial. Speculation that different chemicals result in different shapes of EOG features (Ottoson 1956) has received little support due to the complexity of EOG waveforms. Also, EOG waveforms are affected by mechanical factors such as the positioning of the electrodes and variations of stimulant flow rates.

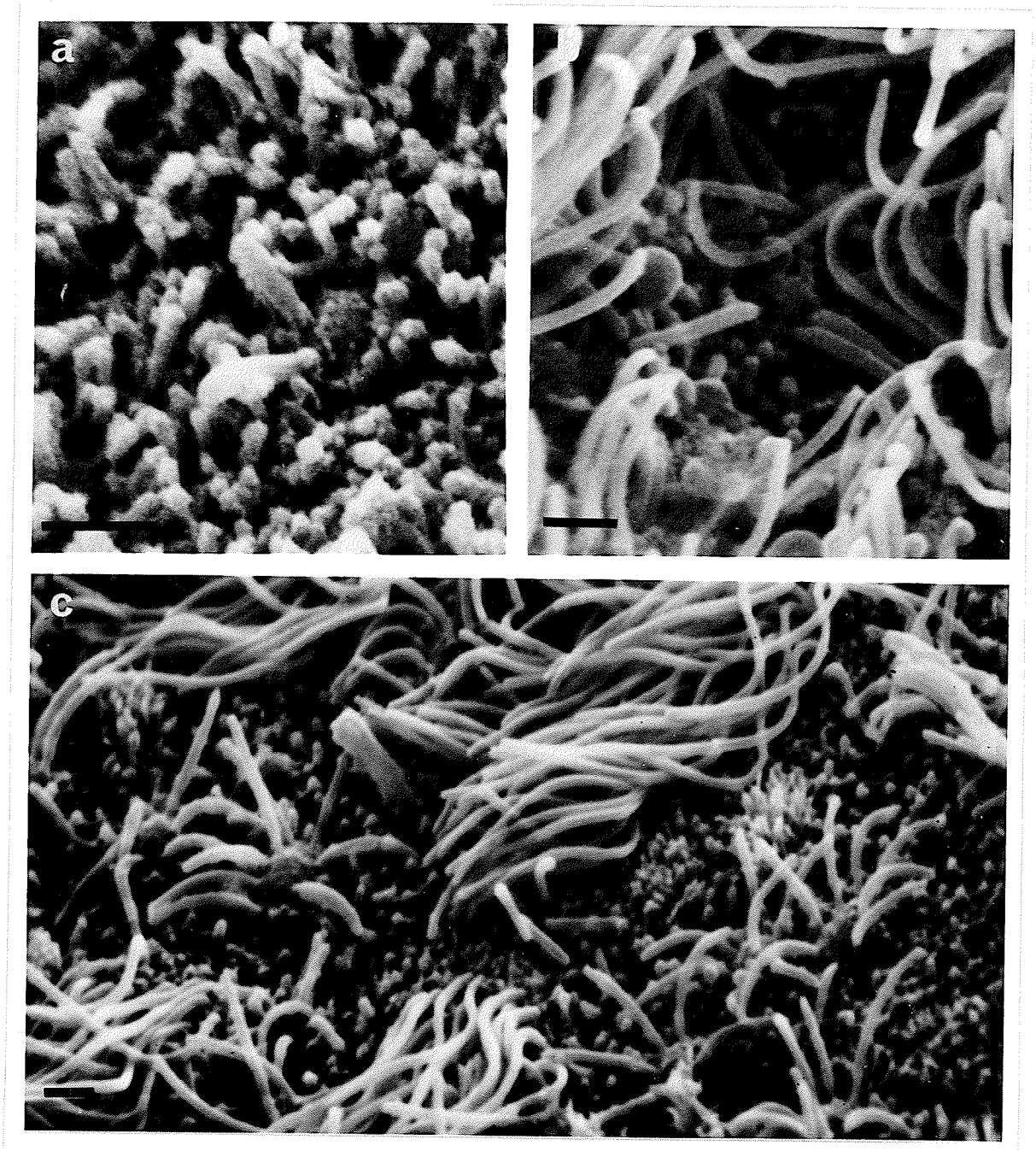


Figure 2.18 Scanning electron micrographs of **(a)** the middle portion of the olfactory placode surface from an stage $E^4$6 lake char, **(b)** the middle portion of the olfactory placode surface from an stage $E^5$8 embryo, **(c)** a portion of the olfactory epithelium surface from a posterior lamella in a juvenile lake char of 20 cm in total length. Bar = 1 μm.

With a well designed stimulation system (Sveinsson 1992) combined with data normalization in the present study, the mechanical influence during EOG recording has been minimized. Thus, it becomes possible to examine whether different chemicals affect EOG features.

My data have shown that the response latency and TPRs of EOGs were statistically different between bile acid and amino acid stimulations, although the EOG traces for these two types of chemicals appeared similar (Fig. 2.2). Recent studies on signal transduction of olfactory neurons have pointed out that the response latency is largely dependent on the speed of second messenger loading in sensory neurons (Firestein and Werblin 1989; Firestein et al. 1990, 1993; Firestein and Shepherd 1991). The magnitude of phasic response of olfactory neurons to stimuli is implicated as a general representation of the maximum generator potential. The magnitude of tonic response is the overall balance of two mechanisms, the continuous activation of the receptor potential and the quick adaptation feedback (Nakamura and Gold 1987; Firestein and Werblin 1989; Breer et al. 1990; Ronnett et al. 1991; Boekhoff and Breer 1992; Dawson et al. 1993; Boekhoff et al. 1994). Therefore, differences in response latency and TPRs of EOG features for bile acids and amino acids may be derived from the distinct signal transduction mechanisms between these two classes of stimuli.

By comparing the sign of the electric current and estimating maximum response magnitude of the whole cell recording to that of the EOG in the salamander *Ambystoma tigrinum*, Lowe and Gold (1991) suggested innate similarity between two

types of recordings. The present quantitative EOG feature analysis has demonstrated that there is a good agreement on the time of response latency and the time of rising phase of odorant induced membrane current between EOG and whole cell recordings (Firestein and Werblin 1989; Firestein et al. 1990, 1993; Firestein and Shepherd 1991; Ivanova and Caprio 1993). These consistencies support the idea that EOG is a measurement of the aggregated performance of a large population of generator potentials (Ottoson 1971). The phasic and tonic responses of EOG reflect the activity of stimulus-receptor interactions.

Olfactory sensitivity and specificity for bile acids

Present EOG results have demonstrated that bile acids are a group of potent olfactory stimulants for lake char. This observation is consistent, in principle, with EOG data in rainbow trout (Hara et al. 1984), zebrafish (Michel and Lubomudrov 1995), sea lamprey (Li et al. 1995) and olfactory bulb recording in grayling and Arctic char (Dϕving et al. 1980). However, this study in lake char is the examination focused on structure-activity relationships and receptor specificity for bile acids as olfactory stimuli in teleosts.

Stimulus-receptor interaction properties

Self-adaptation is comparable to ligand-binding in the presence of full agonists, which provides an ideal environment to study the physicochemical properties of stimulus-receptor interaction (e. g. Boeynaems and Dumont 1980). The present

observation that C-R curves shift parallel to the right along the logarithmic scale up to adaptation concentration during self-adaptation is similar to the finding in lobster *Homarus americanus* NH_4 receptor cells (Borroni and Atema 1988), but different from CYS self-adaptation in Arctic char (Sveinsson and Hara 1990a). Present self-adaptation results have indicated that the interaction of bile acids with their receptors is in a truly competitive and fully reversible manner at the concentration range tested. The results also suggest that olfactory responses to bile acids may be mediated by specific receptors. The constant K_B during self-adaptation at different concentrations indicates that neither negative, nor positive cooperativity was involved between bile acid and receptor interactions. These rational bases are important to interpret cross-adaptation data based on the law of mass action.

Prediction of olfactory responses during cross-adaptation

Cross-adaptation is useful in investigating the involvement of single or multiple receptors mediating an observed electrophysiological response. Though it seems obvious that the concentration and the potency of adapting compounds affect the degree of inhibition to test stimuli, different experimental strategies and the complicated biological system in *in vivo* studies sometimes make it difficult to interpret cross-adaptation results (Beets 1973; Baylin and Moulton 1979; Mair 1982; Zack-Strausfeld and Kaissling 1986). For example, it has been reported that two chemicals with similar concentrations or equipotencies may have different inhibitory power (Cain and Engen 1969; Caprio and Byrd 1984). In some cases, more potent compounds

have higher adaptation effects on test stimuli (Sugimoto and Sato 1981; Ohno et al. 1984). By contrast, it has been concluded that there is no correlation between a compound's potency and the cross-adaptation power for olfactory responses (Baylin and Moulton 1979). Moreover, adaptation may induce enhanced responses to test substances (Borroni and Atema 1989). Present data have shown that strong stimulants are better adapters in general for compounds of the same group (e.g. TLS *vs* TCS). However, a weak stimulant may have higher inhibitory effects than some powerful stimulants from other groups (e.g. CA *vs* TLS). Therefore, observed adaptation effects on self-adaptation and cross-adaptation in the present study are predictable on the basis of ligand-binding principle. Self-adaptation is a special case of cross-adaptation when the full agonist (the stimulus itself) is used as an adapting compound. In cross-adaptation, when the adapting compound is from the same group as the test stimuli, the potency of the stimulus determines the degree of inhibition. Partial reduction of responses to a bile acid (e.g. TLS) induced by an adapting compound from other bile acid groups (e.g. CD or TCA) in this study can be interpreted under the circumstance that the adapting chemical acts as a partial agonist to compete for receptors that are for other groups of bile acids, because self-adaptation has shown that the bile acid-receptor interaction is fully reversible, and because response summations of bile acid binary mixtures across the groups have excluded the suspicion of physical interference among bile acids. It is not unexpected that a bile acid may interact with more than one type of bile acid receptor, if there are multiple bile acid receptors, because molecular structures between groups of bile acids are similar. The

present results have shown conclusively that the adaptation strategy I used to analyse interactions of bile acids with their receptors in the olfactory epithelium is appropriate. Applications of traditional ligand-binding methods to analyse physiological responses of olfactory neurons may lead us to a better understanding of the mechanism of olfactory discrimination.

Bile acid receptors and structure-activity relationships

Present results have shown that if bile acids tested are arranged into three groups based on C-R relationships, that is free, amidated and sulphated bile acids, each group behaves differently in many aspects of stimulus-receptor interactions. (1) C-R relationships of EOG phasic responses and TPRs were different among three groups of bile acids. (2) EOG responses were completely inhibited during adaptation to other members of the same group, but only partially affected during adaptation to compounds of other groups. (3) Cross-adaptation decreased the slopes of C-R curves but changed little on the response thresholds for chemicals of other groups (e.g. Fig. 2.6), in contrast to parallel shift of C-R curves found in self-adaptation. (4) A weak stimulus within same group was a better adapter than a more potent stimulus of other groups. (5) Olfactory responses to either TCA, TLS or PZS remained during adaptation to binary, trinary or a four-compound mixture from different groups. (6) Olfactory responses to a binary mixture of bile acids across groups were the summation of responses to individual components in the mixture. Responses to a binary mixture could be larger than either of the individual stimuli at equal molarity

(Fig. 2.12, mixture responses of TCA and CD).

It can be concluded from the above results that olfactory responses to bile acids are mediated by several distinct receptors and/or transduction mechanisms. These bile acid receptors are specific for 1) free bile acids, with no substituents at C_{24} side chain nor sulphation on sterol ring hydroxyls, 2) amidated bile acids, with N-acyl amidation, but not hydroxy sulphation, and 3) sulphated bile acids, with one or more hydroxy sulphation on sterol rings. The likelihood of another type of bile acid receptor for PZS-like bile acids is also indicated (Fig. 2.11).

The structure-activity relationship analysis of bile acids with different substituents and cross-adaptation results suggest that hydroxyl sulphation or side chain configuration is critical for individual receptor recognition. The numbers, and more importantly, the position and orientation of hydroxyls (but not the oxo groups) presented on the ring structure affect the stimulus effectiveness. Replacing hydroxyls with oxo groups decreases the stimulant potency. The rigid cholanic backbone is necessary for maintaining the molecular stereo structure (bile acids *vs* gonadal steroids), although functional substituents are important for stimulus-receptor interactions. Moreover, the side chain is an important participant in stimulus-receptor interactions, for shortening or lengthening the side chain markedly reduces bile acid effectiveness as olfactory stimulants (e.g NDC and CE).

With regard to the functional structure of bile acids in terms of its stimulatory potency, it appears that an α- axial stereo hydrophilic face formed by hydroxyls of 7α- and/or 12α-, together with atoms at C_{24} (Barnes and Geckle 1982; Barns 1984;

Carey 1985), is important. They render bile acids potent as olfactory stimuli. Epimerization of 7α-hydroxyl has changed the hydroxyl from the axial to the equatorial, which prevents 7-hydroxyl interacting with the binding locus, and an additional 6-hydroxyl could alter spatial positions of 7 α- or 3α- hydroxyl atoms. Both reduce the stimulant potency (TUC < TCD; HC < CD; THD < TLC). These features suggest that bile acids interact with their receptors in a point-to-point fashion rather than the collective effects of the hydrophilic hydrogen bond and the electrostatic forces of molecules. Additional support is from the fact that the most hydrophilic and most hydrophobic bile acids are both ineffective as olfactory stimulants (HD and THD *vs* CN and TCN).

Free and amidated bile acids have a great similarity in the structure-activity relationships. This explains why amidated bile acids have greater inhibitory effects than sulphated bile acids on free bile acid stimulation. The detection thresholds for free bile acids are lower than those for amidated equivalents. This, together with cross-adaptation and binary mixture results, suggests the presence of specific receptors for free bile acids. Glycine amidated bile acids are more potent inhibitors than taurine amidates on free bile acid stimulation. This may be due to glycine amidated bile acids bearing a shorter and less charged side chain substituent than their taurine amidates.

The most effective chemical to interact with sulphate bile acid receptors in this study is TLS. Both taurine amidation and the 3α-hydroxyl sulphation are important for its potency. Substituents other than 3α-sulphate on the sterol rings may not be required to interact with sulphated bile acid receptors. However, substituents at C_7

and C_{12} positions may be important for sulphated bile acids to bind to other bile acid receptors. Modification of the 7α- or 12α-hydroxyl (TCS, DCS, CAS, UCS and CDM) is not favourable for interactions with free and amidated bile acid receptors. This explains why the maximum response for TCS is less pronounced than that for TLS, and why DCS or CAS is far less stimulatory than DC or CA. The most stimulatory bile acids in both amidated and sulphated groups are in taurine conjugated forms. This structural similarity suggests that TCA may moderately interact with the sulphated bile acid receptors, and that TLS with amidated bile acid receptors. The presence of a 3α-hydroxyl in free and amidated bile acids may be effective for these bile acids to interact with sulphated bile acid receptors.

This is the first study to demonstrate the presence of different bile acid receptors in the teleost olfactory system and to determine the optimal substrate for each type of receptor. The results appear similar to the primary report in sea lamprey (Li and Sorensen 1994). They suggested there are four types of bile acid receptors in the sea lamprey olfactory epithelium. The present study has demonstrated that a relatively independent bile acid receptor responding best to PZS-like bile acids is also present in the lake char olfactory system. Taurocholic acid is a potent olfactory stimulant to other salmonids and several cyprinids (ref. Chapter 1). It seems that bile acid receptors, like amino acid receptors, exist in many fish species. It is yet to be determined if similar receptors present in a wide range of species are simply a result of evolution or also have significance in chemical signal detection. By comparing olfactory responses to bile acids in lake char and rainbow trout (Zhang and Hara,

unpublished), together with data from sea lamprey (Li et al. 1995), it appears that the receptor specificity is similar among species. However, conformational or configurational differences may occur for the same type of receptor in different species. For example, rainbow trout are much more sensitive to GLS than lake char. This suggests some differences of sulphated bile acid receptor structures in the two species. Furthermore, lake char and rainbow trout are more sensitive to 5β- forms of bile acids, while sea lamprey to 5α- forms (CA *vs* ACA). With this kind of receptor diversity, each species could acutely sense one or a few compounds among a group of stimulus at relatively low thresholds, which may be important for fish.

It seems that lake char are more sensitive to free bile acids than other species. Olfactory detection thresholds for free bile acids are lower in lake char than in rainbow trout (Zhang and Hara, unpublished). In zebrafish, the olfactory sensitivity to CD and TCA are at similar range (Michel and Lubomudrov 1995). By contrast, in lake char olfactory thresholds for CD is one logarithmic unit lower than that for TCA. Whether the high olfactory sensitivity of lake char to free bile acids is critical for conspecific communication is an interesting question for future study.

Relationships with other olfactory stimuli

Bile acids, amino acids, PGs and gonadal steroids are four major classes of olfactory stimuli for fishes (ref. Chapter 1). Even though fish are sensitive to over 30 naturally occurring amino acids, olfactory responses to amino acids are mediated by CYS-like and ARG-like receptors (ref. Hara 1992c, 1993, 1994a, b; Chapter 1). The

PGF represents most, if not all, of PGs examined as the olfactory stimuli (Sorensen et al. 1988). Present reciprocal cross-adaptations have demonstrated that olfactory responses to bile acids are mediated by receptors and transduction mechanisms distinct from those for amino acids and prostaglandins. In salmonids, olfactory responses to gonadal steroids are limited to a few C-19 steroids (Hara and Zhang 1996; Zhang and Hara, unpublished). Gonadal steroid 17,20P is neither stimulatory, nor an antagonist to bile acids in lake char. Chances of cross reaction of stimulus-receptor between bile acids and other gonadal steroids is unlikely, for the cholanic side chain is required to interact with bile acid receptors. The finding that receptors for bile acids are independent of those for gonadal steroids and PGs is similar to observations in goldfish (Sorensen et al. 1987, 1988) and in Arctic char (Sveinsson 1992), where EOG responses to TCA remained or unchanged during adaptation to 17,20P or PGF. It can be concluded that there are distinct receptors and transduction mechanisms in the fish olfactory system to detect bile acid signals.

Detection of bile acids appears important to fish. The information on recognition and discrimination of bile acids mediated by specific receptors in the olfactory epithelium is transmitted and coursed to central projection (Sveinsson 1985). After being encoded by secondary neurons in the olfactory bulb (Døving et al. 1980; Hara and Zhang 1996a; b), signals for bile acids are further transmitted to higher station of the central nervous system (Sorensen et al. 1991a). These processes appear totally independent of those for amino acids (Sorensen et al. 1991; Hara and Zhang 1994, 1996). The separate information transmission from peripheral receptors to

central projections between bile acids and amino acids suggests the importance and fundamental functional differences of these two classes of odorants to fish.

Consistent with studies on Arctic char and grayling (Døving et al. 1980), bile acids are more potent than amino acids for olfactory stimulation in lake char (Table 2.1; Hara et al. 1993). However, olfactory responses to most bile acids saturate at low concentrations (Fig. 2.4). Amino acids at relative high concentrations, e. g. at 10^{-4} M, may elicit responses with a greater magnitude than bile acids (Erickson and Caprio 1984). Therefore, one has to be careful when ranking the stimulatory effectiveness by screening response magnitudes of various compounds at a single concentration. For a better understanding of the effectiveness of various compounds, it is desirable to look at the entire profile of C-R relationships of stimuli and/or the underlying transduction mechanisms, as has been suggested by Hara (1982b). A comparison of the response magnitude at a single concentration will provide us with useful information when these compounds interact with the same receptors.

Olfactory responses to bile acids at different life stages

It has been suggested that circulating hormonal level may affect olfactory responses to certain stimulants. The olfactory sensitivity to some compounds may change during a particular stage of life such as spawning or smoltification in some species (Moore and Scott 1991; Morin and Døving 1992; Bjerselius and Olsén 1993; Irvine and Sorensen 1993; Morin et al. 1995). Olfactory responses were affected by steroid hormone treatments (Hara 1967; Oshima and Gorbman 1968, 1969; Cardwell

et al. 1995). No indication of this type of change has been observed in terms of threshold and response magnitude in response to bile acids in the spawning or prespawning lake char tested. However, this result itself does not exclude the role of bile acids during spawning. Prostaglandins are pheromones for Arctic char, yet the olfactory sensitivity to PGs is not different between juveniles and spawning adults (Sveinsson 1992, Sveinsson and Hara 1995). Moreover, the olfactory sensitivity to sex pheromones 17,20P and 17,20P-S in goldfish provides no indication of sexual differences or temporal changes (Sorensen et al. 1987, 1995; Sorensen 1992). An understanding of the underlying mechanisms of the olfactory facilitation is required to discuss the relationship between olfactory facilitation and pheromone detection.

One of the most interesting findings in this study is that lake char are selectively sensitive to bile acids at early developmental stages. The following factors argue against the speculation that olfactory responses to bile acids may not be receptor specific and simply due to detergent-like effects: 1) the concentrations of bile acids used in the experiments are far below the toxic or detergent levels (Schölmerich et al. 1984), 2) the spike activity was monitored concurrently with the emerging of sensory neurons in lake char embryo, 3) olfactory responses to bile acids were concentration dependent, and 4) the embryonic rainbow trout at similar stages responded to amino acids but not to bile acids (Zielinski and Hara 1988; Zhang and Hara, unpublished).

Salmonids, like most other teleosts, have two types of morphologically distinct sensory neurons (ref. Yamamoto 1982; Zieske et al. 1992). On the basis of the presence of two kinds of sensory neurons and spatial distribution of bulbar responses

to two classes of stimuli, bile acids and amino acids, Thommesen (1983) hypothesized that CSNs are specific to bile acid-like substances and MSNs to amino acids. Although the present study has shown that the CSNs may be responsible for olfactory sensitivity to bile acids in embryonic lake char, this result does not necessarily support Thommesen's hypothesis. In lake char, the time course of CSNs and MSNs appearance, and their developmental sequence are ontogenetically similar to that in rainbow trout (Zielinski and Hara 1988). The CSNs are shown to respond to amino acids in rainbow trout (Zielinski and Hara 1988). This morphological similarity and physiological diversity of olfactory neurons between embryonic lake char and rainbow trout strongly indicate that sensory neurons responsive to bile acids and amino acids may be CSNs. It also suggested that MSNs may be sensitive to amino acid stimulation in rainbow trout (Zielinski and Hara 1988, Hara and Zielinski 1989). Sea lamprey, bearing only ciliated neurons (VanDenbossche et al. 1995), respond to both amino acids and bile acids (Li and Sorensen 1992; Li et al. 1995). These observations suggest that functional diversity of olfactory neurons is not necessarily associated with morphological differences of sensory neurons. The temporal difference of the olfactory sensitivity to these two distinct classes of stimuli, however, suggest that receptors for bile acids and amino acids may reside on separate ciliated neurons.

The biological significance of the acute sensitivity to bile acids in embryonic lake char requires investigation. In postnatal rat, exposure to an odour stimulus results in expression of the odorant dependent marker protein in the olfactory bulb

(Guthrie and Gall 1995). Something similar may occur in embryonic lake char. If lake char embryos develop in a bile acid-rich environment, functional differentiation of olfactory neurons may be induced by bile acids and express olfactory responses to bile acids earlier than to other stimuli. Whether lake char eggs contain bile acids should be considered in future studies, as it has been speculated that lake char eggs are a source of chemical attractants for spawning lake char (Martin 1960; Foster 1985). Bio-active compounds, such as steroid and thyroid hormones, are found in fish eggs (Kobuke et al. 1987; Tagawa and Hirano 1987; de Jesus et al. 1991; Schreck et al. 1991; Hwang et al. 1992). Hormones in the eggs are of maternal origin (Schreck et al. 1991; Hwang et al. 1992). Bile acids may have been accumulated in lake char eggs during oocyte maturation, alternatively, they may be introduced into eggs during fertilization, as enhanced bile acid excretion in the urine of prespawning male lake char was observed (Chapter 5). The ability of early detection of bile acids could be important for lake char in connection with their homing, kin recognition, or spawning behaviour.

CONCLUSION

This chapter has presented extensive data to show that bile acids are potent olfactory stimuli to lake char. The olfactory sensitivity and specificity for bile acids are mediated by specific receptors and transduction mechanisms that are distinct from those for other known olfactory stimuli. Differences of the response latency and the

TPRs of EOG waveforms reflect the different transduction mechanisms between bile acids and amino acids. Among bile acids tested, the most potent bile acids are CD, DA, ODA, TLS, CA, TCS, HC, TCD and TCA, with detection thresholds at sub-nanomolar concentrations. At least three types of bile acid receptors are responsible for olfactory responses to these most stimulatory bile acids. They are free, amidated and sulphated bile acid receptors. These receptors are characteristic and relatively independent. However, a bile acid may react with one or more bile acid receptors, depending on the concentration. The specificity and multiplicity of receptors in the olfactory epithelium indicate that fish have the ability to detect and discriminate different quality and quantity of bile acids, as well as the composition of mixtures in a chemical world they live in. This ability seems to be maintained during the entire life of lake char. Also, olfactory responses to bile acids emerges earlier than to amino acids during early developmental stages of lake char. The biological significance of which in relation to lake char homing, kin recognition or mating, is yet to be determined.

CHAPTER 3

Characterization and Localization of Bile Acid Receptors by Autoradiographic Binding

INTRODUCTION

Electrophysiological studies using EOG recording have suggested that olfactory sensitivities to bile acids in lake char are specific and mediated by at least three types of bile acid receptors that are distinct from those for amino acids, prostaglandins and gonadal steroids (Chapter 2). To demonstrate that bile acid receptors characterized electrophysiologically represent true membrane-bound receptors, further biochemical characterization is required. Binding assays using sedimentable fractions of olfactory cilia or olfactory epithelial membranes to study amino acid receptors have been successful in fishes (Cagan and Zeiger 1978; Cancalon 1978; Novoselov et al. 1980; Rhein and Cagan 1980, 1983; Fesenko et al. 1983; Rhein 1983; Rehnberg and Schreck 1986; Lo et al. 1991). However, Brown and Hara (1981, 1982) have pointed out that those sedimentable fractions as used by these authors are a complex mixture of cell membrane components and binding of amino acids is likely to be interrelated with amino acid transport systems. They further suggest that inconsistencies between various aspects of amino acid binding (or accumulation) by the sedimentable fraction of olfactory rosettes and electrophysiological responses preclude unequivocal

characterization of binding as olfactory receptors. Using a similar method, Lo et al. (1994) failed to show tissue specificity of TCA binding in Atlantic salmon.

Receptor autoradiography provides another useful method for direct analysis of specific binding and receptor localization. Receptor autoradiography represents the combination of traditional neuroanatomy with more recent developments in neuropharmacology. The method has high reproducibility and is 3-5 orders of magnitude more sensitive than regular binding assays (Kuhar 1985; Kuhar et al. 1986; Kuhar and Unnerstall 1990). The development of ligand autoradiography has greatly advanced our knowledge of receptor localization in brain and other tissues. Because of several drawbacks on applications of regular binding assays in binding studies for olfactory receptors (Lancet 1986, 1988), the autoradiographic method was applied in the present study to characterize bile acid receptors that satisfy the established criteria for receptors, and to localize specific binding in the olfactory epithelium.

MATERIALS AND METHODS

Experimental animals

Lake char of 34-45 cm total length were raised and maintained as described in Chapter 2.

Cleaning of slides and coverslips

The slides and coverslips used in this experiment were cleaned by soaking

overnight in a chromic-sulphuric acid cleaning solution (Fisher Chromerge), rinsed profusely in tap water for 3 hrs, followed by a 3-hr rinse with distilled water. The cleaned slides and coverslips were then placed in slide boxes and dried in an oven.

Tissue preparation

Each fish was anaesthetized and immobilized as described in Chapter 2. The anaesthetized fish was secured in a holding apparatus placed in a plexiglass trough. Water containing MS222 was perfused through the mouth and over the gills. Olfactory rosettes were exposed by removing the dorsal aspect of skin and the cartilage of olfactory sacs. After perfusing nares with plain water for several minutes (see below), the fish was sacrificed by decapitation. The rosettes were excised and embedded in Tissue-TEK II O.C.T (Division Miles Laboratories, IN, USA) and mounted on a microtome chuck. Tissue sections were cut at 20 μm on a Cryo-cut microtome at -20°C (American Optical, NY, USA), thaw-mounted onto acid washed slides, and stored at -100°C.

For deciliated rosette tissue preparation, the olfactory cilia were removed by "ethanol-calcium shock" before sacrifice (Kashiwayanagi et al. 1988). Briefly, the epithelia were perfused with 5 mM phosphate buffer solution (pH 8.0), containing saturated ethyleneglycol-bis-(β-amino-ethyl ether) N.N'-tetra-acetic acid (EGTA), 10% ethanol and 0.2 M sucrose, at 400 ml/min for 1.5 min, followed by 100 mM $CaCl_2$ at 100 ml/min for 10 min. The rosettes were then washed with plain water at 100 ml/min for 10 min before used for tissue sections. In control rosette tissue preparation

(normal rosette tissue), the "ethanol-calcium shock" was replaced by perfusing water only.

The olfactory neuron degenerated rosette (NervX) was prepared by severance of olfactory nerves anterior to olfactory bulbs as described by Brown and Hara (1981). Olfactory responses to 10^{-5} M SER and 10^{-6} M TCA were abolished at all sites on the epithelium when examined 18-day post-axotomy. The NervX used in the experiments were 20-day post-axotomy rosettes.

Tissue sections of deciliated rosette, NervX, and other tissues for the examination of tissue specificity (midbrain, lateral muscles, upper portion of the intestine and liver) were prepared and stored as mentioned above.

Chemicals

[^{3}H(G)]Taurocholic acid ([^{3}H]TCA) (sp. act.2.0 Ci/mmol) was purchased from Dupont Canada, ON, Canada, with purity of 98.2%. The purity was re-examined before experiments by high performance liquid chromatography (HPLC) analysis on a 3-μm bead size Adsorbosphere HS C_{18} column, eluting with 0.01 M potassium phosphate (pH 7.5)-acetonitrile (75:25) at 1.0 ml/min. Over 94% of the radioactivity was resolved at the retention time for the TCA peak. Other chemicals used for experiments were purchased from Sigma Chemical or Fisher Scientific, ON, Canada, at analytical grade or at the purest form available.

Receptor characterization

The experimental protocol for receptor characterization was based on Kuhar and Unnerstall (1990). Slide-mounted tissue sections were brought to room temperature and incubated in Coplin jars containing 32 nM [^{3}H]TCA and then washed in buffer to remove non-specific binding (operationally defined). A final quick in-out dip in distilled water was to remove buffer salts. All incubation and washes were processed in an ice bath. Phosphate buffer (50 mM, pH 7.2) was used for incubation and washes. The incubated tissue sections were then wiped off with half pieces of Whatman glass microfibre circles (GF/B, 2.4 cm) and subjected to scintillation counting for radioactivity. Radioactivity losses due to TCA adhering to Coplin jars and slides were not detected as examined by counting the radioactivity of incubation solution before and 2.5 hrs after incubation of blank slides ($100\pm1.1\%$).

Total binding and non-specific binding (operationally defined) of rosette tissues were determined by incubating tissue sections in [^{3}H]TCA in the absence or presence of 100 μM unlabelled TCA, respectively. For liver tissues, 1 mM unlabelled TCA was used to obtain non-specific binding. The specific binding was determined by subtracting non-specific binding from total binding, and expressed in terms of picomole or counts per minute (cpm) bound [^{3}H]TCA per milligram of dried tissue sections. An autobalance (d=0.001 mg, Perkin-Elmer, CT, USA) was used to weigh the dried tissue sections. Saturation and competition data were analysed by EBDA/LIGAND programs for IMB microcomputer (McPherson 1985). The fitting model and its parameters chosen were based on optimal F test and Runs test.

Receptor mapping

Autoradiograms were generated according to the method of Kuhar and Unnerstall (1990). Emulsion coated coverslips were obtained by dipping acid-washed coverslips (Corning No.1, 25x77 mm) into Kodak NTB-3 emulsion (diluted 1:1 with Millipore filtered water) at 43°C. The coated coverslips were air dried and stored with desiccants at 3°C for late use.

Rosette tissue sections were incubated in 32 nM [^{3}H]TCA for 90 min and washed with buffer for 5 min, an optimal incubation condition based on binding assays (see Results section). The sections were then dried by cold dry air and apposed to NTB-3 emulsion coated coverslips. The coverslip was glued to the frosted end of the slide. Two teflon sheets (3 mm thick, about 30x50 mm size), one on the top of the coverslip, and the other on the bottom of the slide were held together with the slide-coverslip complex by a clip binder. The labelled assemblies were then wrapped with desiccants in plastic bags and light proof aluminum sheets, and stored in a refrigerator (3°C).

After 200-day of exposure, the binder and teflon sheets were removed in the dark and the coverslip was gently bent away from the tissue sections with a spacer. The emulsion was developed in Kodak D-19 film developer (1:1) for 4 min, placed in Kodak liquid hardener (diluted 1:13 in water) for 15 s, fixed in Kodak Rapid Fixer for 5 min, and rinsed in water for 20 min. The tissue sections were then rapidly fixed with picro-acetate fixative and stained with the modified haematoxylin-eosin (Robert 1966) method (Chayen et al. 1969). Finally the spacer was removed and the coverslip

was mounted with Permount on top of the tissue sections.

Four types of rosette tissue sections were prepared for autoradiogram generation: 1) normal rosette tissue sections for total binding (NRT), 2) normal rosette tissue sections for non-specific binding (NRN), 3) deciliated rosette tissue sections for total binding (DRT), and 4) deciliated rosette tissue sections for non-specific binding (DRN). These four preparations were processed in a same manner, except that an additional 100 μM of unlabelled TCA was added to incubation jars for non-specific binding sections. The autoradiograms were semiquantified by the JAVA video analysis program (Jandel Scientific, CA, USA). The grey value of grains (GV) on the coverslips due to tritium exposure was the reciprocal of the grey level (GL) of transmitted light over the grains minus the reciprocal of the GL of the background (Beck and Rainbow 1985). The grain density (GD) was the number of grains in unit area. To minimize the variation caused by different emulsion thickness and developing conditions on individual slides, the relative grey intensity (RGI) and the relative grain density (RGD) were used. The RGI was defined as the ratio of GV for the sensory epithelium area and the GV for the adjacent connective tissue area. Similarly, the RGD was the ratio of the GD for epithelium area and the GD for the adjacent connective tissue area. Four different locations on one section of each slides and a total of six slides were measured for each preparation. Differences in the RGI and the RGD among preparations were analysed by one way ANOVA and Tukey's HSD tests (SYSTAT program). Probability $P < 0.05$ was considered significantly different. The data are presented as means and standard errors in the graphs.

Kinetic analysis of electrophysiological responses

To examine the biological relevance of receptors in this study, the dissociation constant (K_D) of binding was compared to that from kinetic analysis of EOG data. Olfactory responses to TCA were obtained by EOG recording as described in Chapter 2. Because olfactory responses to bile acids complies with the law of mass action (Chapter 2), the data are valid for kinetic analysis using the occupation equation. A linear multiple receptor model (Hara 1982b) was adopted to estimate the K_D for bile acid receptors based on electrophysiological data. As further suggested by Sveinsson and Hara (1990a), a correction factor, C, was introduced in the model to compensate for the responses to the control stimulus (plain water):

$$R = [B_{max\,1} \cdot S/(K_{D\,1}+S)] + \cdots + [B_{max\,n} \cdot S/(K_{D\,n}+S)]+C \qquad [3.1]$$

where R is the response, S is the concentration, and B_{max} is the binding capacity. Numbers 1 to n denote different receptors. Non-linear regression was generated by the SigmaPlot program (Jandel Scientific).

RESULTS

Dissociation curve

The time course of dissociation of [^{3}H]TCA binding was examined by washing rosette tissue sections with buffer at different time periods after 90-min incubation in 32 nM [^{3}H]TCA. Figure 3.1 shows that the non-specific binding declined quickly at first and then decreased linearly, beginning at about 3 min. A 5-min wash was used

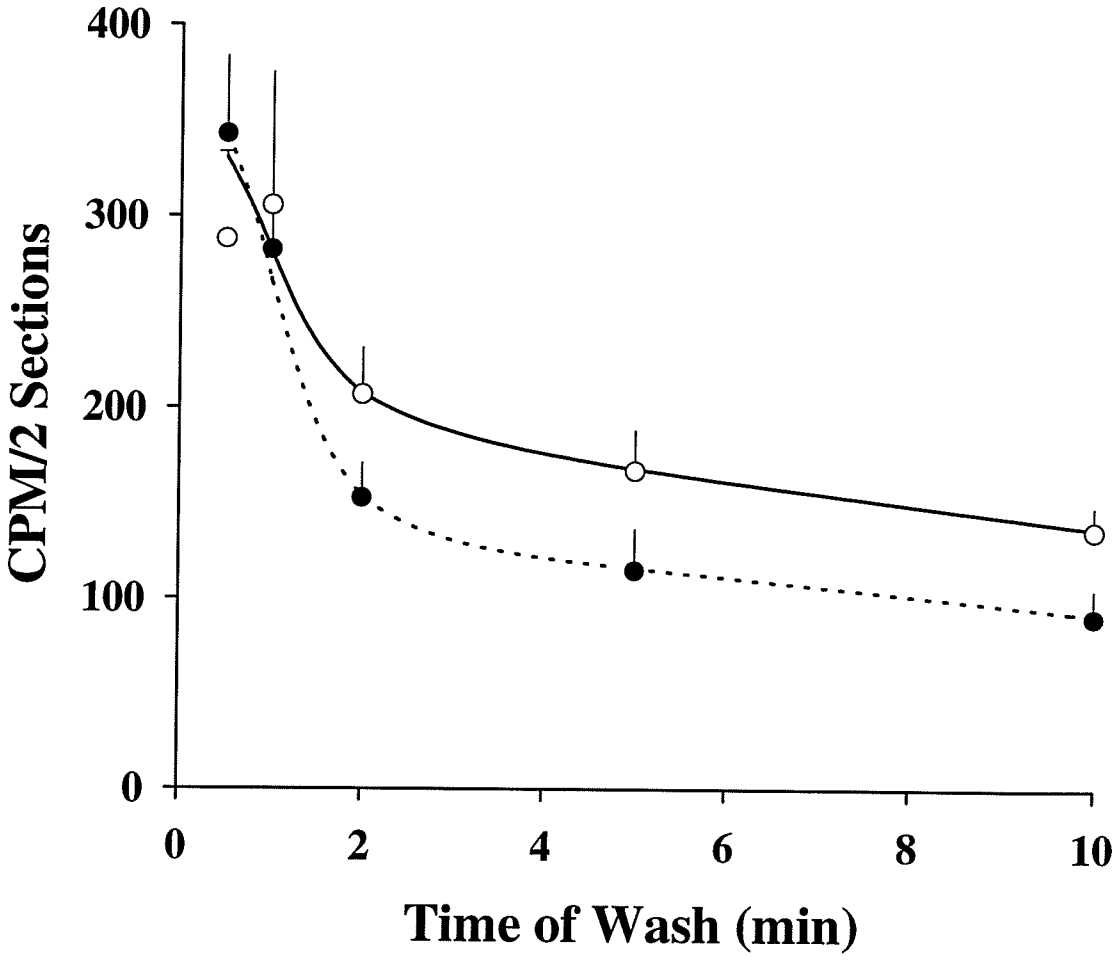


Figure 3.1 Time course of dissociation of [^{3}H]taurocholic acid binding to rosette tissue sections. (○) total binding; (•) non-specific binding, determined as the binding in the presence of an additional 100 μM of unlabelled taurocholic acid in incubation jars.

in the following experiments.

Association curve

Rosette tissue sections were incubated in 32 nM [^{3}H]TCA for 30, 60, 90 and 120 min and washed for 5 min to examine the association of [^{3}H]TCA to receptors. The total binding increased in the first 60 min and remained steady in the next 60 min (Fig.3.2). Therefore, unless otherwise specified, tissue sections incubated in 32 nM [^{3}H]TCA for 90 min and washed for 5 min were employed as the working condition in the following study.

The receptor reversibility was examined by adding 100 μM of unlabelled TCA in the incubation jars when the binding association reached a steady state. Figure 3.2 shows that the total binding was reduced to the non-specific binding level in about 10 min after unlabelled TCA was introduced.

Competition

Displacement of [^{3}H]TCA binding due to the presence of unlabelled TCA at increasing concentrations from 1 nM to 1 mM in incubation jars is shown in Figure 3.3a. The concentration-response data were analysed by EBDA/LIGAND programs on a microcomputer. A two-site model fitted the data at a significant level (P=0.000). The K_D for the high-affinity binding site was 110 nM and for the low-affinity site was 34 μM. The B_{max} was 4 pmol/mg for the high-affinity site and $3x10^2$ pmol/mg for the low-affinity binding site.

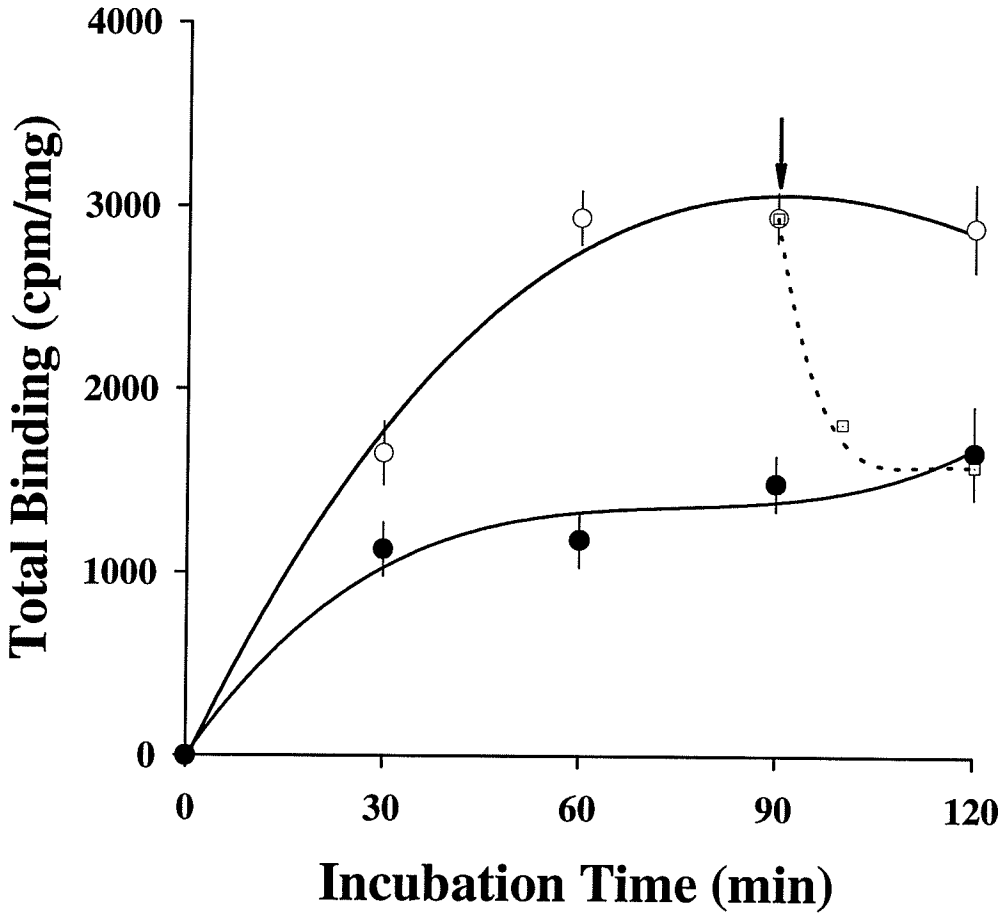


Figure 3.2 Association and reversibility of [^{3}H]taurocholic acid binding to rosette tissue sections. (○), total binding; (•), non-specific binding, determined as the binding in the presence of an additional 100 μM unlabelled taurocholic acid in incubation jars. Reversibility (□) is demonstrated by adding 100 μM unlabelled taurocholic acid to [^{3}H]taurocholic acid incubation jars when binding equilibrium was reached (indicated by ↓).

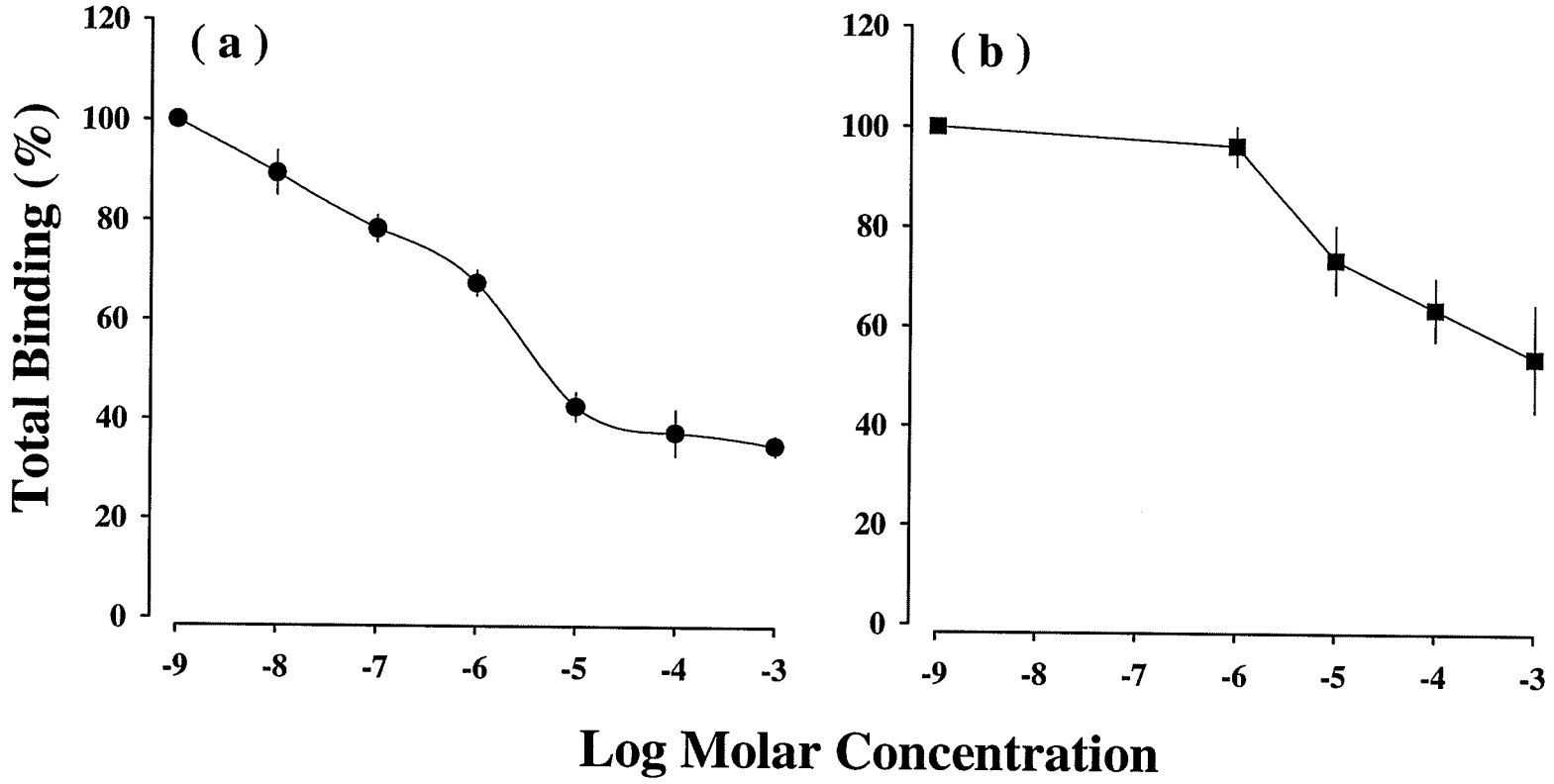


Figure 3.3 Displacement of [^{3}H]taurocholic acid binding in the rosette **(a)** and liver **(b)**, determined by the presence of an increased concentration series of unlabelled taurocholic acid in incubation jars that contained 32 nM [^{3}H]taurocholic acid. Data are presented as percentages of total binding in the presence of 1 nM unlabelled taurocholic acid.

Saturation analysis

The saturation kinetics of TCA was examined by incubating tissue sections with varying concentrations of [^{3}H]TCA at 9-100 nM ranges. The Scatchard plot showed non-linearity of the specific binding (Fig. 3.4). When attempts were made to fit the data first to a single-site model using EBDA/LIGAND programs, the parameters corresponded to the high-affinity site obtained in the competition study (see previous section). However, a further analysis indicated that a two-site model was fitted better for this data set ($P < 0.005$), giving a K_D of 5 nM and B_{max} of $2x10^{-1}$ pmol/mg for the high-affinity binding site. The parameters of the next binding site were 1 μM for K_D and 1x10 pmol/mg for B_{max}.

Tissue specificity

To investigate specificity of [^{3}H]TCA binding to rosette tissues, binding assays were conducted together with other tissue sections, liver, upper portion of the intestine, lateral muscle and midbrain (Fig. 3.5). The specific binding was highest for the rosette tissue, followed by the liver tissue. Specific binding of [^{3}H]TCA to the lateral muscle and midbrain tissues was negligible. Bile acid receptors in the liver tissue were further investigated in comparison with those in the rosette. The competition data indicated that the liver had a low-affinity binding site for [^{3}H]TCA with K_D of 26 μM and B_{max} of $5x10^2$ pmol/mg (Fig. 3.3b).

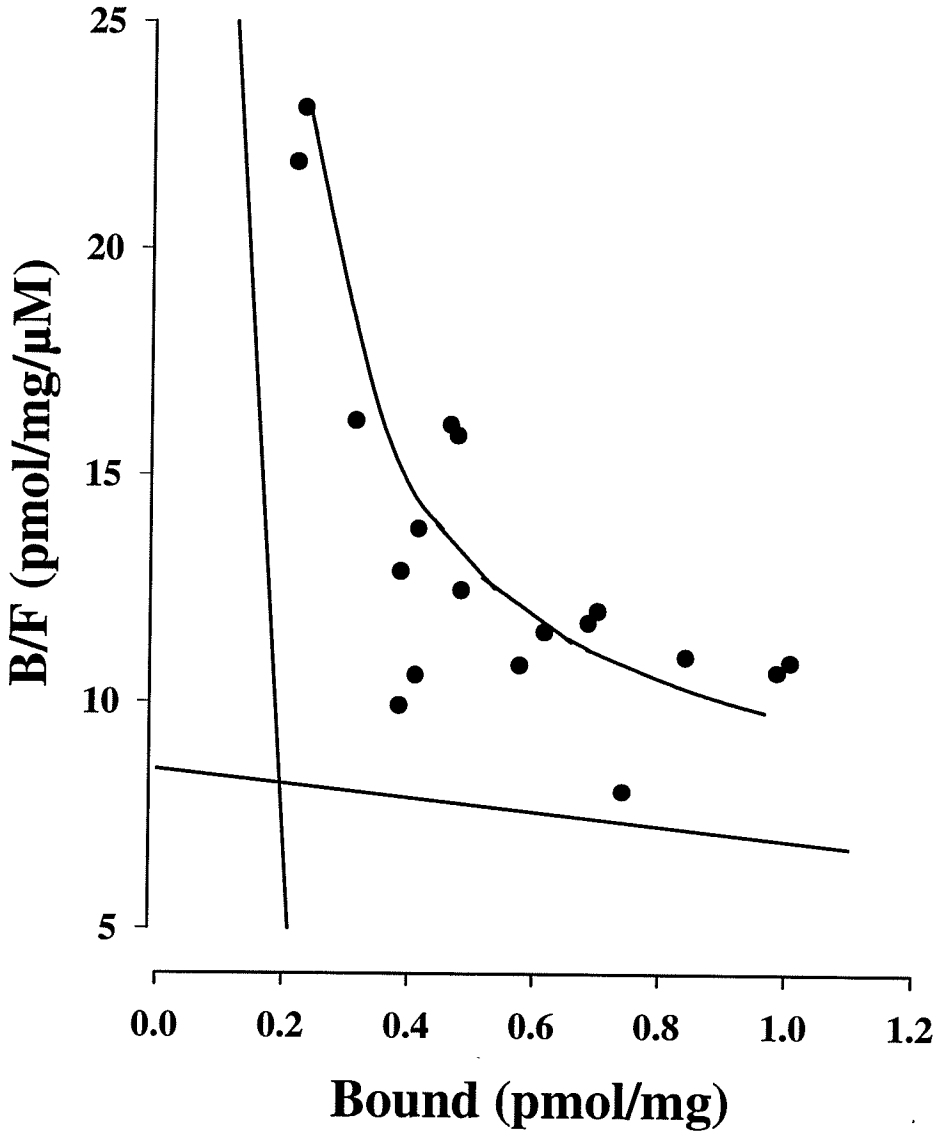


Figure 3.4 Scatchard analysis of specific binding of [^{3}H]taurocholic acid to rosette tissue sections at concentration 9-100 nM and the fitted curves. B/F=bound/free.

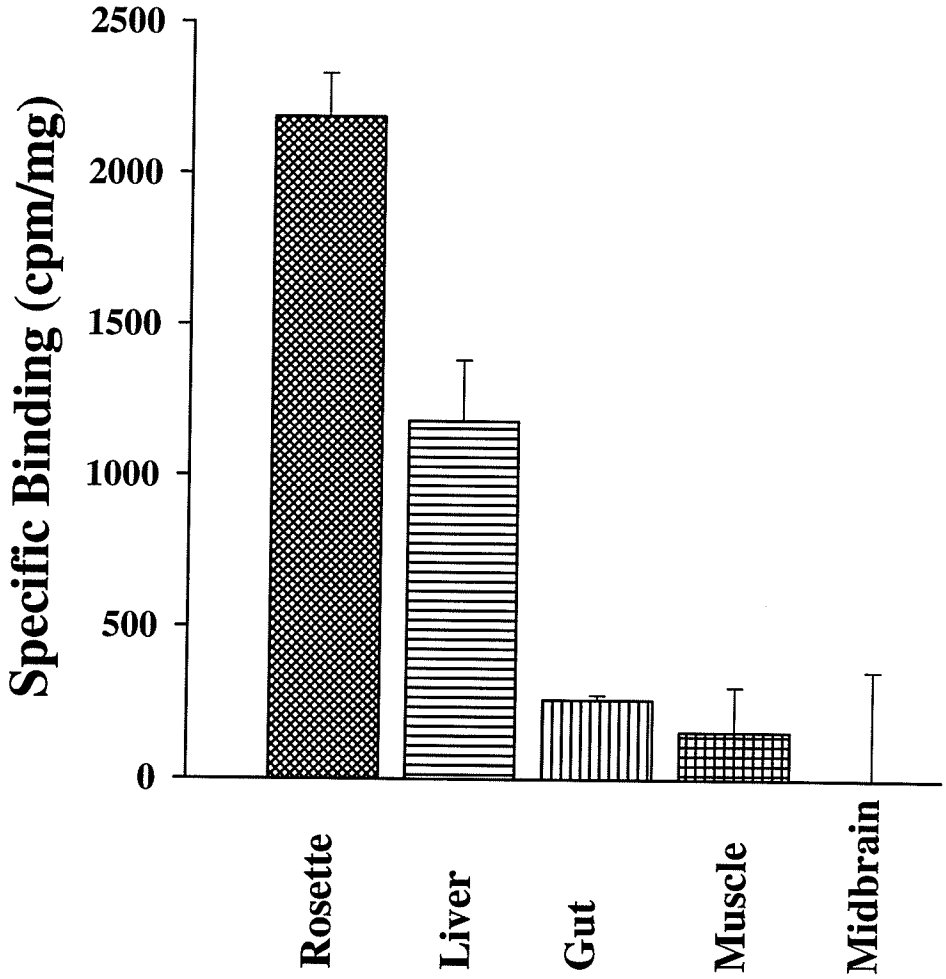


Figure 3.5 Tissue specificity of [^{3}H]taurocholic acid binding. The specific binding presented here is the difference of binding in the presence of 1 nM unlabelled taurocholic acid and binding in the presence of 100 μM unlabelled taurocholic acid.

Pharmacological specificity

Bile acids and an amino acid, CYS, were used in a competition study to examine structure-activity relationships of [^{3}H]TCA binding in rosette and liver tissue sections (Fig. 3.6). Specific binding of [^{3}H]TCA in the rosette was affected by 1μM TCA and TCD, but not by 1 μM CD and CYS, while the inhibitory effect of 1 μM TLS was marginal (Fig. 3.6a). However, a high concentration of TLS notably inhibited specific binding of [^{3}H]TCA to the rosette, and CD had a slight effect (Fig. 3.6b).

The displacement of [^{3}H]TCA binding in the liver by the above five chemicals was different from those in the rosette. Taurolithocholic acid 3-sulphate was the most powerful inhibitor over all, even stronger than TCA itself. At the concentration of 1 μM, TLS reduced specific binding of [^{3}H]TCA to 70% of the original level (Fig. 3.6c). The competitive effects of 100 μM TCA, TCD and CD on [^{3}H]TCA binding in the liver were considerable (Fig. 3.6d).

Location of specific binding

Specific binding of [^{3}H]TCA was compared in three types of rosette tissue sections: normal, deciliated and NervX. The deciliated rosette tissue sections retained about one quarter of specific binding as compared to the normal one (Fig. 3.7). No specific binding was observed in the NervX preparation.

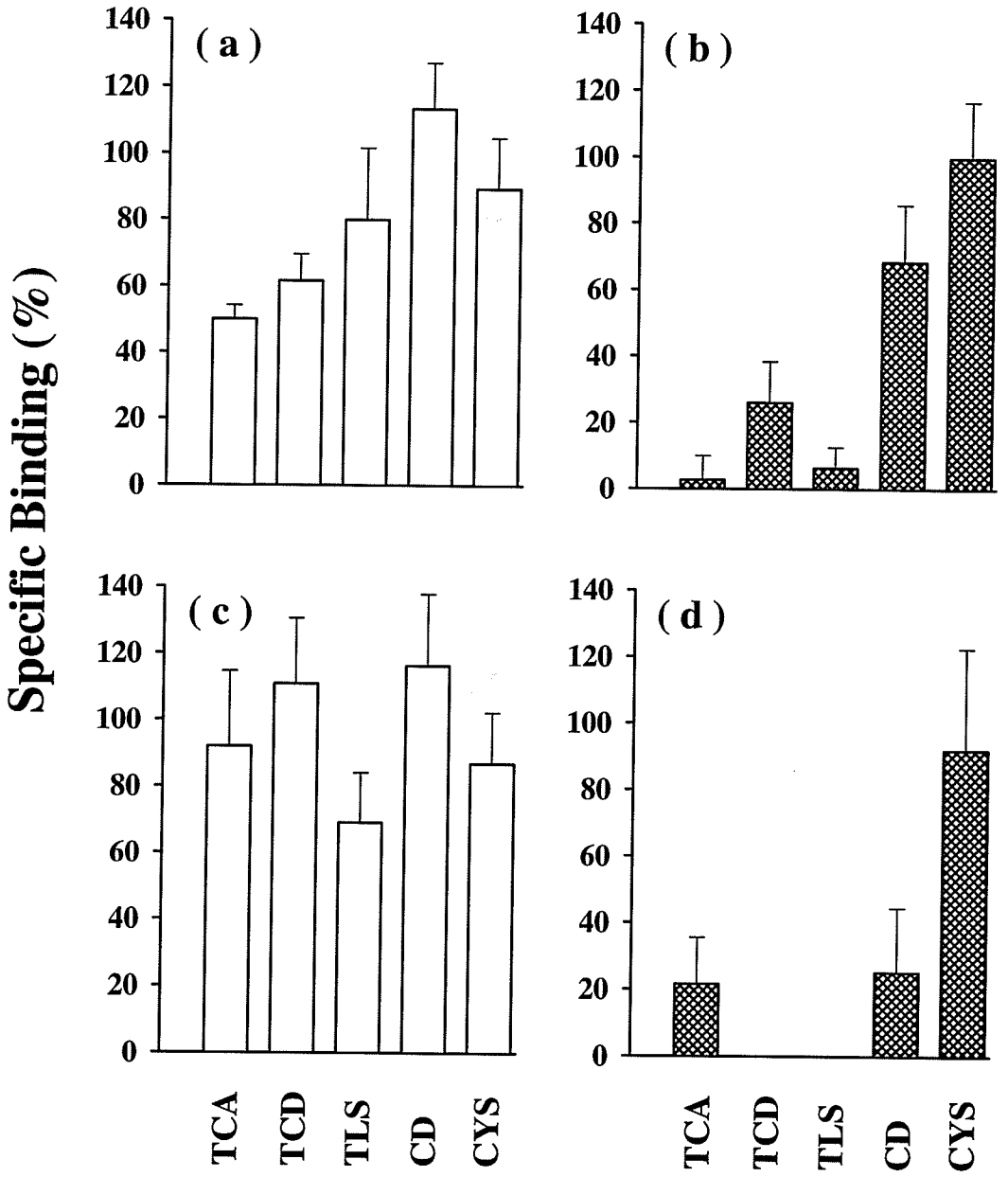


Figure 3.6 Inhibition of [^{3}H]taurocholic acid binding when 1 μM (open bar) or 100 μM (crosshatched bar) of the following chemicals were present in incubation jars: taurocholic acid (TCA), taurochenodeoxycholic acid (TCD), taurolithocholic acid 3-sulphate (TLS), chenodeoxycholic acid (CD) and L-cysteine (CYS). **(a) and (b)**, rosette tissue sections; **(c) and (d)**, liver tissue sections. The data are presented as the percent reduction of specific binding. The specific binding was the total binding minus non-specific binding.

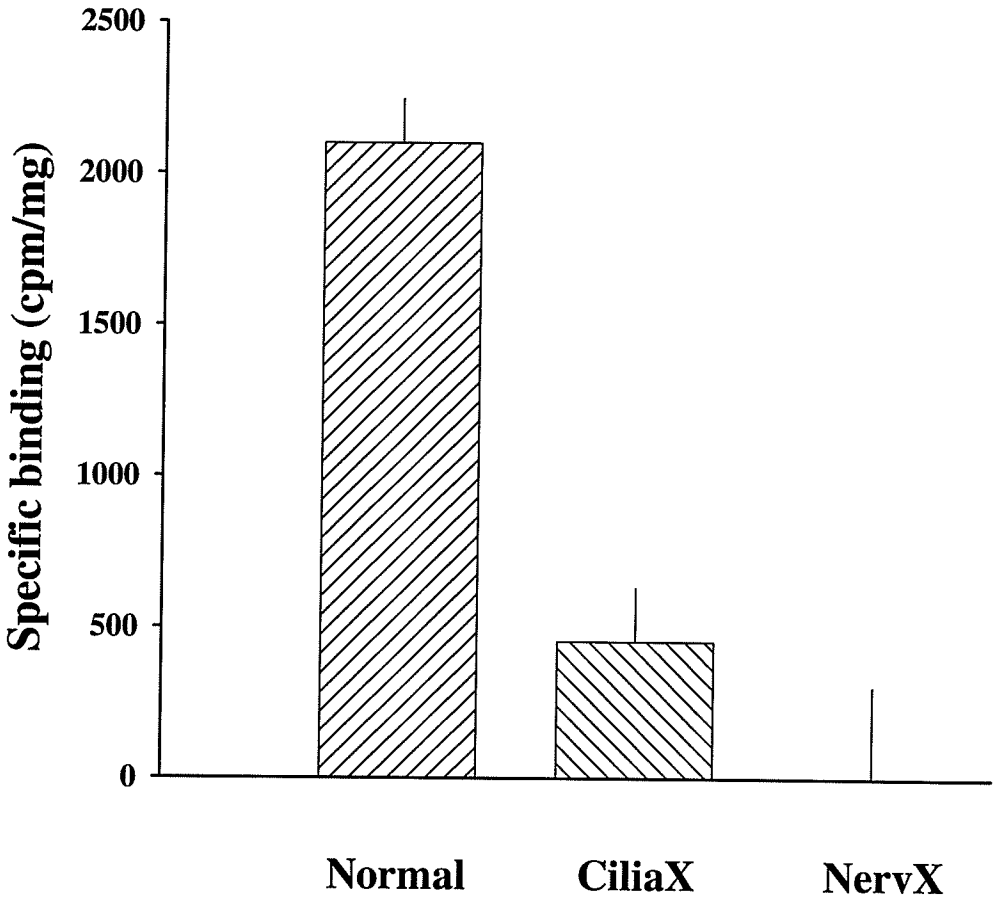


Figure 3.7 The effects of deciliation and denervation on [^{3}H]taurocholic acid binding. The specific binding was the total binding minus non-specific binding. Normal=normal rosette tissue sections; CiliaX=deciliated rosette tissue sections; NervX=denervated rosette tissue sections.

Functional relevance of ligand binding

Figure 3.8 shows relationships between EOG response magnitudes and TCA stimulation at 10^{-10}-10^{-5} M range. Analysis of the C-R relationship using non-linear model of the occupancy equation [3.1] revealed K_D for first two binding sites was 4.7 nM and 0.8 μM, respectively. Another low-affinity binding site at K_D around 30 μM was predicted.

Receptor autoradiograms

After [^{3}H]TCA bound rosette tissue sections were apposed to emulsion coated coverslips, the localized binding activity of sections determined the degree of exposure to emulsions on the coverslip. The density of silver grains on the coverslip due to radioisotope exposure thus represents the binding activity. Autoradiograms show that the highest density of grains was present on coverslips over the sensory epithelium area of the NRT preparation (Fig. 3.9). In the other three preparations, NRN, DRT and DRN, the grain density and intensity on coverslips over sensory epithelium areas were marginal. The RGI and RGD levels were much higher for the NRT preparation than either NRN, DRT or DRN preparations ($P=0.000$), whereas the latter three had similar levels of RGI and RGD ($P > 0.6$) (Fig. 10). The RGI and RGD value of NRN and DRN were close to 1, indicating that the radioactivity of sensory epithelia was similar to that of the adjacent lamina propria in sections for non-specific binding.

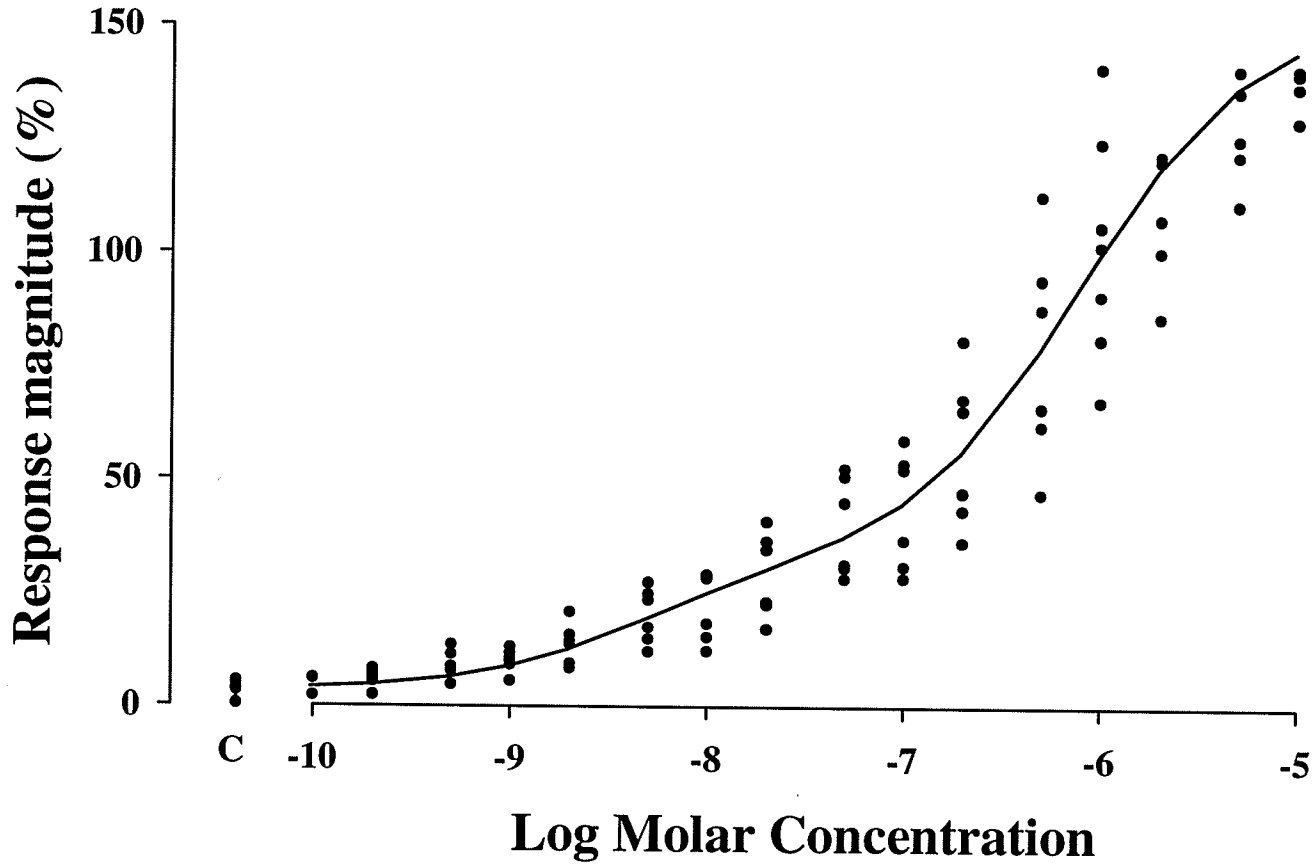


Figure 3.8 Concentration-response relationships of electroolfactogram responses to taurocholic acid and the fitted curve calculated based on occupancy equation. Response magnitudes are normalized as percentages of the 10^{-5} M L-serine responses. C=control response.

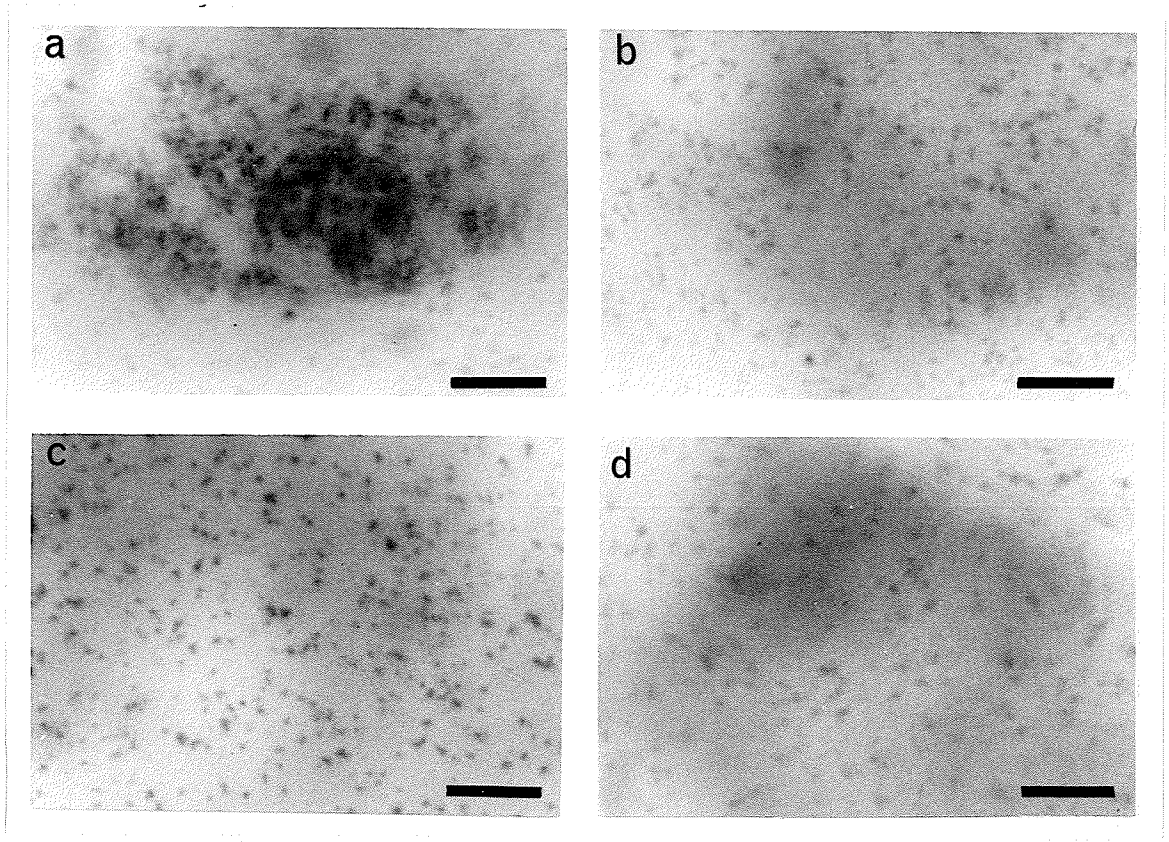


Figure 3.9 Autoradiograms of sensory epithelium portions for four types of rosette tissue preparations, showing different degrees of the grain density and intensity that reflect the binding activity in the olfactory epithelium. **(a)** normal rosette tissue sections for total binding; **(b)** normal rosette tissue sections for non-specific binding; **(c)** deciliated rosette tissue sections for total binding; and **(d)** deciliated rosette tissue sections for non-specific binding. bar = 10μm

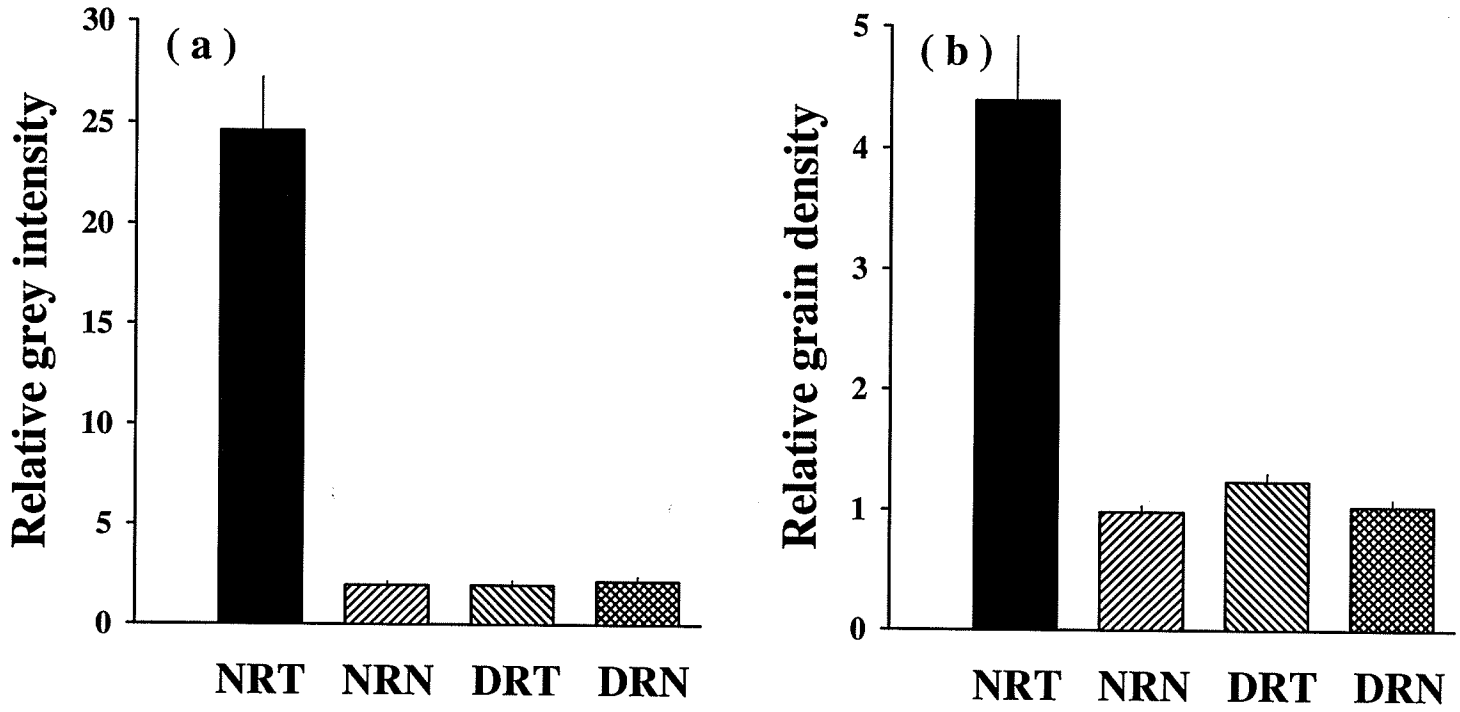


Figure 3.10 The relative grey intensity **(a)** and relative grain density **(b)** for autoradiograms obtained from normal rosette tissue sections for total binding (NRT), normal rosette tissue sections for non-specific binding (NRN), deciliated rosette tissue sections for total binding (DRT), and deciliated rosette tissue sections for non-specific binding (DRN).

DISCUSSION

This study provides the first direct evidence for specific binding and distribution of bile acid receptors in the fish olfactory epithelium. The data have demonstrated the presence of three receptor components for bile acids in the olfactory epithelium with K_D of 5 nM, 1 μM and 34 μM, respectively. These parameters are in an excellent agreement with the dissociation constants derived from electrophysiological responses calculated based on the occupation equation. The chemical specificities of [^{3}H]TCA binding established in this study are in accordance with electrophysiological results (Chapter 2) in that: 1) receptors for bile acids are independent of those for amino acids, 2) TCD interacts with bile acid receptors in a manner similar to TCA, 3) TLS is a partial agonist of TCA, and 4) CD competes weakly with TCA. Therefore, the present receptor autoradiography has identified bile acid receptors that are relevant to olfactory responses to bile acids.

This study has demonstrated that specific binding of TCA in the rosette tissue is directly associated with olfactory sensory neurons in the lamella. Autoradiograms show that the specific binding is limited to the sensory epithelium. The specific binding is further localized on the olfactory cilia because the binding is drastically reduced in deciliated rosette preparations. There are at least three tentative explanations for the residual specific binding after deciliation: 1) deciliation was incomplete, 2) sensory receptors are localized not only on the cilia but also on the distal olfactory knob, and 3) bile acid receptors are distributed to cells other than

olfactory sensory neurons. The fact that the specific binding was absent in the denervated rosette tissues, whose olfactory neurons were degenerated (Burns et al. 1981; Evans et al. 1981; Evans and Hara 1985; Zielinski and Hara 1992), rules out the third possibility. The result that olfactory receptors reside in the olfactory cilia or knob is consistent with the published biochemical and electrophysiological observations. Rhein and Cagan (1980, 1981) have shown that the cilia-membraneous fraction accounts for a great deal of odorant binding. Patch-clamp recordings have provided direct evidence that the initial ligand-binding and signal transduction in response to odorant stimulation take place on the sensory cilia, and possibly the dendrite knob of the olfactory neurons (Nakamura and Gold 1987; Firestein et al. 1990; Lowe and Gold 1991).

Present binding assays have shown that considerable amount of specific binding of [^{3}H]TCA presents not only in rosette but also in liver tissues. The finding that there is significant amount of [^{3}H]TCA specific binding in the liver tissue is not unexpected. Being important steroids for food digestion and many aspects of hepatic organic and anionic transport, bile acids are efficiently taken up from portal blood across the sinusoidal membrane by hepatocytes and secreted into bile (Hofmann 1977, 1994a). Carrier-mediated transport involves binding of bile acids to specific receptors on cell membranes. However, kinetic analysis and pharmacological specificity investigations have revealed that the bile acid receptor in the lake char liver is distinct from those in the rosettes. This low-affinity bile acid receptor in the liver somewhat resembles the receptor found in brush border membranes of the mammalian intestine

in that reducing the number of hydroxyls on bile acid sterol rings enhances the binding affinity (Wilson and Treanor 1977). The binding parameter K_D for the bile acid receptor in lake char liver is in accord with the study on isolated hepatocytes of rainbow trout (Råbergh et al. 1994). The characteristics of this receptor are not comparable to the binding site identified in rat ileal plasma membranes (Simon et al. 1990), nor bile acid uptakes in skate hepatocytes (Fricker et al. 1994).

Judging from binding parameters and pharmacological specificities, all bile acid receptors and transport carriers in the vertebrate digestive system verified to date are different from bile acid receptors in the rosette (e.g. Hoffman et al. 1975; Accatino and Simon 1976; Anwer et al. 1985; Bellentani, et al. 1987; Takacs et al. 1987; Zimmerli et al. 1989; Hardison et al. 1990; Simon et al. 1990; Kramer et al. 1992). The present binding study, together with electrophysiological characterization (Chapter 2), further suggests that bile acid receptors in the char olfactory epithelium differ from bile acid binding sites in serum albumin, serum lipoproteins and intracellular binding proteins found in mammals in two major aspects: the high affinity and stereospecific structure-activity relationships (e.g. Rudman and Kandell 1957; Burke et al. 1971; Roda et al. 1982; Sugiyama et al. 1983; Takikawa and Kaplowitz 1986; Aldini et al. 1987; Takikawa et al. 1987, 1992, 1995; Hedenborg et al. 1988; Pico and Houssier 1989; Farruggia and Pico 1993). Thus, even though TCA binds to other tissues or binding proteins, the bile acid receptors identified in the olfactory epithelium are specific for the olfactory system. It may be of future interest to examine whether bile acid receptors, similar to those in olfaction, are present in organs other than examined.

The present finding that specific binding of [^{3}H]TCA is highest for rosette tissues contrasts with the report by Lo et al. (1994). In their [^{3}H]TCA binding study of Atlantic salmon olfactory rosettes using a plasma membrane enriched preparation, they showed that the [^{3}H]TCA binding was not specific for rosettes. In addition, fractions from the muscle had the highest specific binding for [^{3}H]TCA, while specific binding was similar among fractions of the rosette, liver and brain, and approximately half of that in the muscle (Lo et al. 1994). Although a large amount of binding was observed in muscle tissue sections in the present study, the binding was almost completely undisplaceable by 100 μM unlabelled TCA (Fig. 3.5). The reason for this discrepancy remains open for discussion. Species difference is a plausible explanation. However, different techniques employed in the binding assays should not be ignored. Bile acids are shown to bind to cytosol binding-proteins, Golgi apparatus, endoplasmic reticulum and other subcellular components of mammalian hepatocytes (Strange et al. 1979; Goldsmith et al. 1983; Suchy et al. 1983; Simion et al. 1984; Lamri et al. 1988; Hofmann 1994a). It is presently unknown whether these elements in other tissues have the same properties. Considering that the plasma membrane enriched fraction is a heterogeneous mixture of membranous vesicles, mitochondria, cilia and endoplasmic reticulum (Brown and Hara 1981), a higher concentration of these membrane mixtures in muscles is likely, as compared to other tissues.

This study is the first attempt to characterize and localize fish olfactory receptors by the autoradiographic binding method. The sensitivity of this method (Kuhar 1985) has compensated for, to some extent, the relatively low specific activity

of the ligand used and has overcome some obstacles on biochemical characterization of olfactory receptors (Lancet 1986). The use of tissue sections instead of plasma membrane fractions allows ones to study biochemical properties of receptors close to physiological conditions. Using this method, it becomes possible to visualize the distribution of ligand-receptor binding in the fish olfactory system. The similarity of binding parameters for the bile acid receptor in the liver between this study and the published data (Råbergh et al. 1994) and the excellent agreement between this binding study and electrophysiological results (Chapter 2), warrant the use of receptor autoradiography for future studies on biochemical properties of fish olfactory receptors.

In summary, the present autoradiographic binding study has revealed the presence of multiple bile acid binding components that meet receptor criteria in the lake char olfactory epithelium. These bile acid receptors are highly specific and different from those in the vertebrate digestive system. Specific binding is observed in the olfactory sensory epithelium. The binding activity depends on the receptors located at olfactory sensory neurons, particularly at the cilia. Bile acid receptors in the olfactory epithelium have binding parameters equivalent to those obtained from kinetic analysis of electrophysiological data, and have similar characteristics as those demonstrated in Chapter 2. Thus, bile acid receptors demonstrated in this study are most likely the ones that mediate olfactory responses to bile acids in lake char.

CHAPTER 4

Electrophysiological and Behavioural Responses to Bile Acid Related Natural Excretions

INTRODUCTION

Salmonids are attracted to water scented with some metabolites in natural excretions from conspecifics. Both Arctic char and Atlantic salmon show preference behaviour to substances in juvenile bile, intestinal contents or the urine of their own species (Selset and Døving 1980, Olsén 1987, Stabell 1987, Moore et al.1994). Spawning lake char choose spawning sites rich in conspecific faeces (Foster 1985). Although the chemical identity of these cues are unknown, it is suspected that bile acids are the putative components for these behaviour reactions. In fish, bile acid transformation due to bacteria in the intestine is insignificant (Sacquet et al. 1979; Chapter 5). Bile acid components in the intestine, therefore, should be similar to those in bile. In this chapter, I examine behavioural and electrophysiological responses to bile and urine to seek possible connections between the natural excretion and the effects of bile acids on fish behaviour.

MATERIALS AND METHODS

Fish maintenance

Test fish (total length 10-15 cm) were age 1^+ lake char from the same source as described in Chapter 2. They were maintained in a white coloured glass tank, a background similar to that of the experimental trough, and fed in the evening to avoid any conditional linkage between feeding and the behaviour under investigation.

Electrophysiological studies

Olfactory responses of fish to bile and urine freshly collected from conspecific juveniles were determined by EOG recording as described in Chapter 2.

Procedures for behavioural experiments

Behavioural tests were conducted in a two-way choice behaviour trough, 114 cm in length and proportional to the drawing in Figure 4.1. When filled, the water depth was 9 cm. Plain water (10.5-12.0°C) was delivered to sections A and B at equal flow rate (1 l/min) from a common reservoir and overflowed to waste with two outlets located at section C. Water bodies from sections A and B were well separated along the midline before the efflux as evidenced by dye tests. Dashed lines in Figure 4.1 indicate the locations of three perforated plastic plates. Plastic plates in sections A and B were designed to prevent fish entering water inlets, and to buffer the water turbulence. The plastic plate in section C formed a gate to temporarily restrict

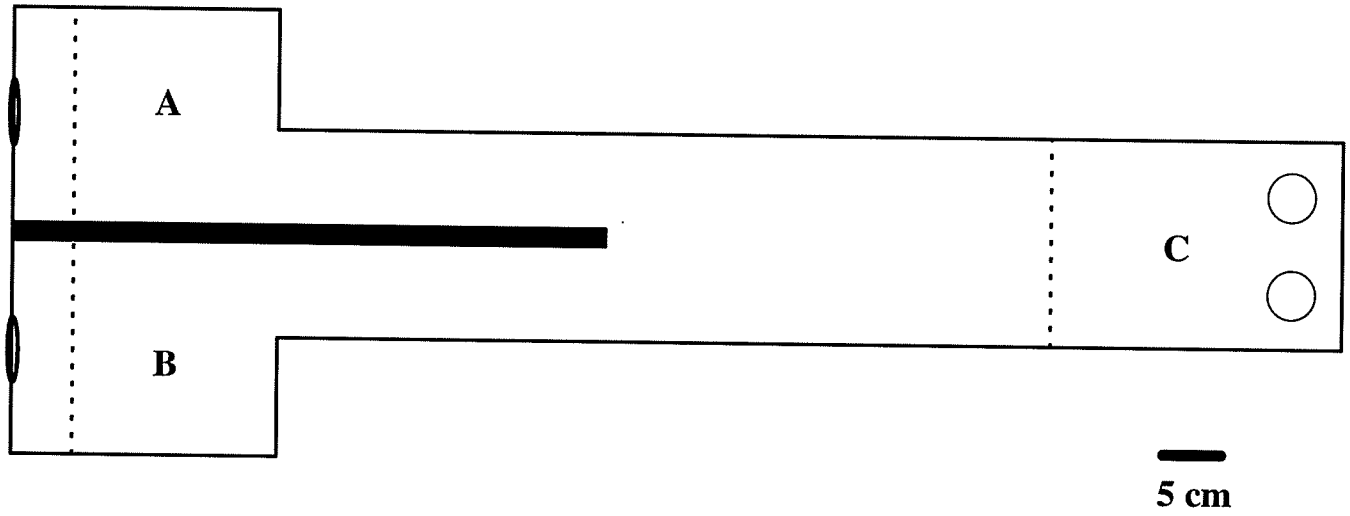


Figure 4.1 Top view of a two-way choice apparatus for behavioural experiments. The diagram is proportional to the actual size. The trough is arbitrarily labelled as sections A, B, and C for the convenience of illustration. Two circles in section C indicate positions of two water outlets. Dashed lines indicate the positions of three perforated plastic plates. Two elliptical circles indicate water inlets. Black bar in the middle of trough indicates the plexiglass wall. Bar=5 cm.

fish at one end of the trough during acclimation.

The behaviour trough was located in an isolated dark room with two fluorescent lamps (32 Watts) at the ends, 120 cm above the trough. Fish locomotion was recorded by means of a video camera.

Bile and urine freshly collected from fasted conspecific juveniles were diluted with distilled water and then pumped into an inlet of either section A or B at a speed of 3.3 ml/min to yield the desired concentration. The other section received the same amount of distilled water delivered in a same manner. The stimulatory section was alternated after each test. The stimulant was well mixed with plain water before it entered the trough. Dye tests indicated that visible dye reached section C within 4.5 min after pumping. The dye colour deepened quickly in the next 1 min.

Four lake char were used in each trial because it has been reported that a single fish often respond abnormally to olfactory stimuli (Olmsted 1918). Fish were introduced to the experimental trough in the evening one day before testing to allow acclimation. The test was conducted during 13:00-15:00 hr. The plastic plate in section C was lifted quietly by a remote string at 4.5 min after the stimulant delivery started. Fish locomotion during the next 20 min was recorded and analysed. The frequency of entry to section A and B was counted. The ratio of entry to the stimulatory section over the total were plotted semi-logarithmically against concentrations of the stimulant. The data obtained from trials of the same stimulatory concentration were pooled and compared with a 0.5:0.5 distribution using the χ^2 test, assuming that random choice (neutral reaction) results in expected frequency of an

equal number of entry to both sections. Correction for continuity was applied in χ^2 test if the sample size less than 200 (Sokal and Rohlf 1981). Probability $P < 0.05$ indicated significant departures from neutral reaction hypothesis.

After each trial, fish were moved to another identical tank so that the same fish would not be used for tests in the next two weeks. The trough was carefully washed, drained, scrubbed with methanol, and finally rinsed with plain water for a few hours before the next test.

RESULTS

Electrophysiological responses

Bile from conspecifics of similar size was a potent olfactory stimulant to lake char. Electrophysiological thresholds for bile determined by EOG recording were at concentrations of 1-5 nl/l dilution (Fig 4.2). To examine if the high olfactory sensitivity to bile was caused by bile acid components, I conducted cross-adaptation experiments. The olfactory sensitivity of lake char to bile was reduced by 1 logarithmic unit or more during adaptation to 10^{-6} M TCA. Response thresholds to bile were also clearly affected during adaptation to 10^{-7} M TLS. However, the inhibitory effect of either 10^{-7} M CD or 10^{-3} M CYS on olfactory responses to bile was minor. Interestingly, although detection thresholds for bile remained unchanged during adaptation to 10^{-3} M CYS, a reduction of response magnitude to bile became notable at the concentration of 100 nl/l dilution and higher.

Figure 4.3 is a semi-logarithmic plot of C-R relationship of EOG responses to

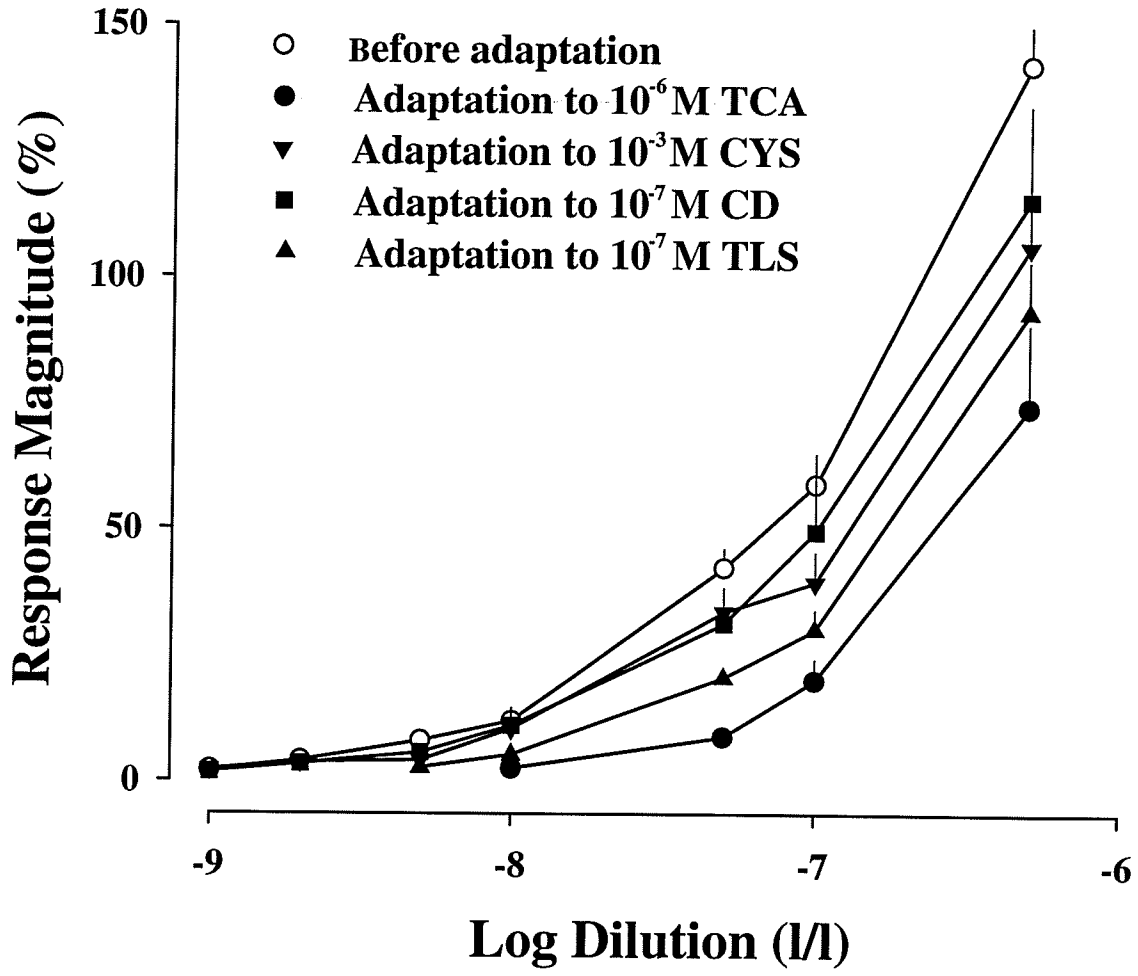


Figure 4.2 Electroolfactogram responses of lake char to conspecific bile at different dilutions before (open symbol) and during (filled symbols) adaptation to various compounds. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. TCA=taurocholic acid; TLS=taurolithocholic acid 3-sulphate; CD=chenodeoxycholic acid; and CYS=L-cysteine.

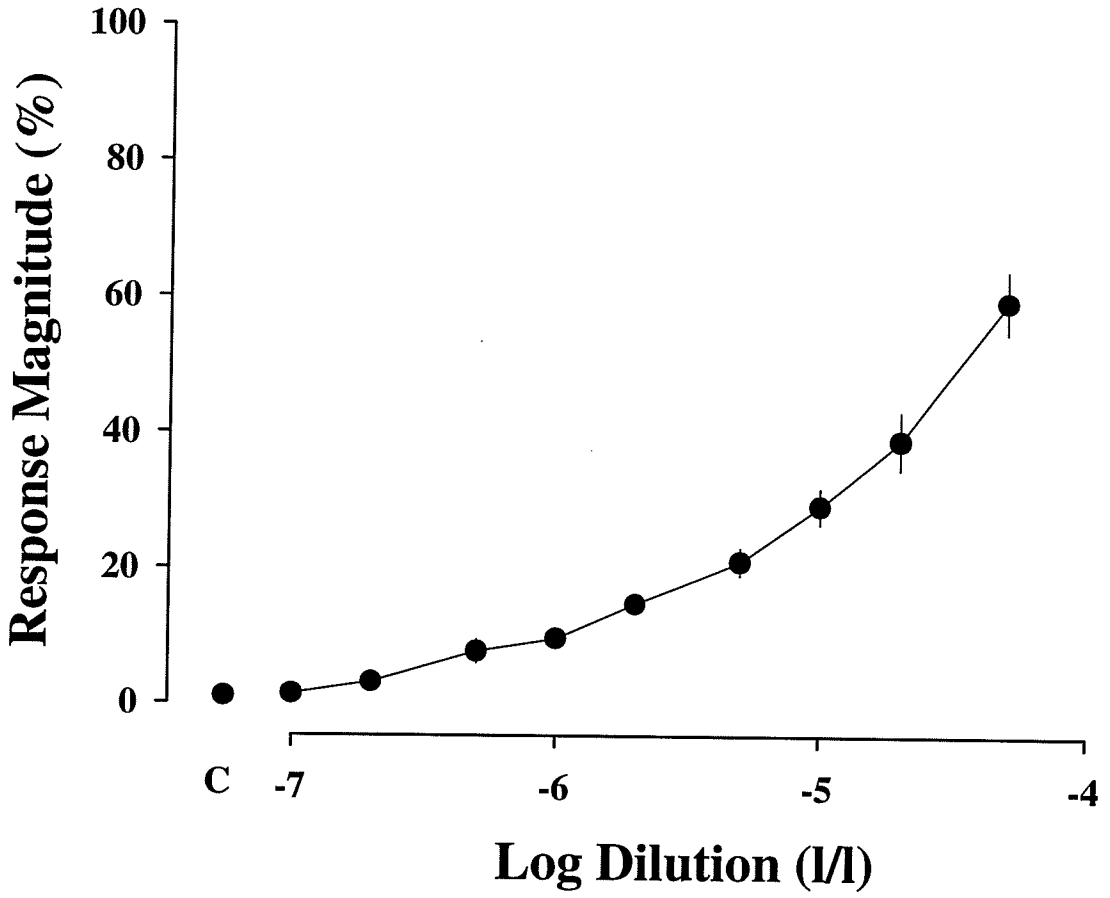


Figure 4.3 Concentration-response relationships of electroolfactogram responses to urine collected from conspecific juveniles. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. C=control response.

urine. Thresholds of olfactory responses to urine were at 0.1-0.5 μl/l dilution. The deviation of response magnitudes for the urine collected from three different fish was small.

Behavioural responses

When both sections A and B received 3.3 ml/min distilled water (control), fish entered either section A or B randomly. The total entry was 386/353, showing no difference to a neutral reaction assumption ($P > 0.1$).

Results of behaviour reactions to bile from conspecifics are shown in Figure 4.4. Lake char chose the section containing bile at dilution above 0.1 nl/l. Behaviour reactions to bile were concentration dependent. However, bile at concentrations below 0.1 nl/l was repulsive to fish.

Fish showed significant preference responses to authentic bile acid TCA at 10^{-8}-10^{-7} M (Fig. 4.5). Behaviour thresholds were at 10^{-9}-10^{-8} M. Avoidance behaviours were not observed at a lower concentration.

When tested with urine at 10 μl/l, fish visited the section containing stimulants more frequently than the control section (63%, $P < 0.01$). In 3 out of 7 trials, it was observed that fish occasionally attacked one another (aggressive behaviour) during the urine stimulation.

DISCUSSION

Behavioural and physiological responses of lake char to bile and urine indicate

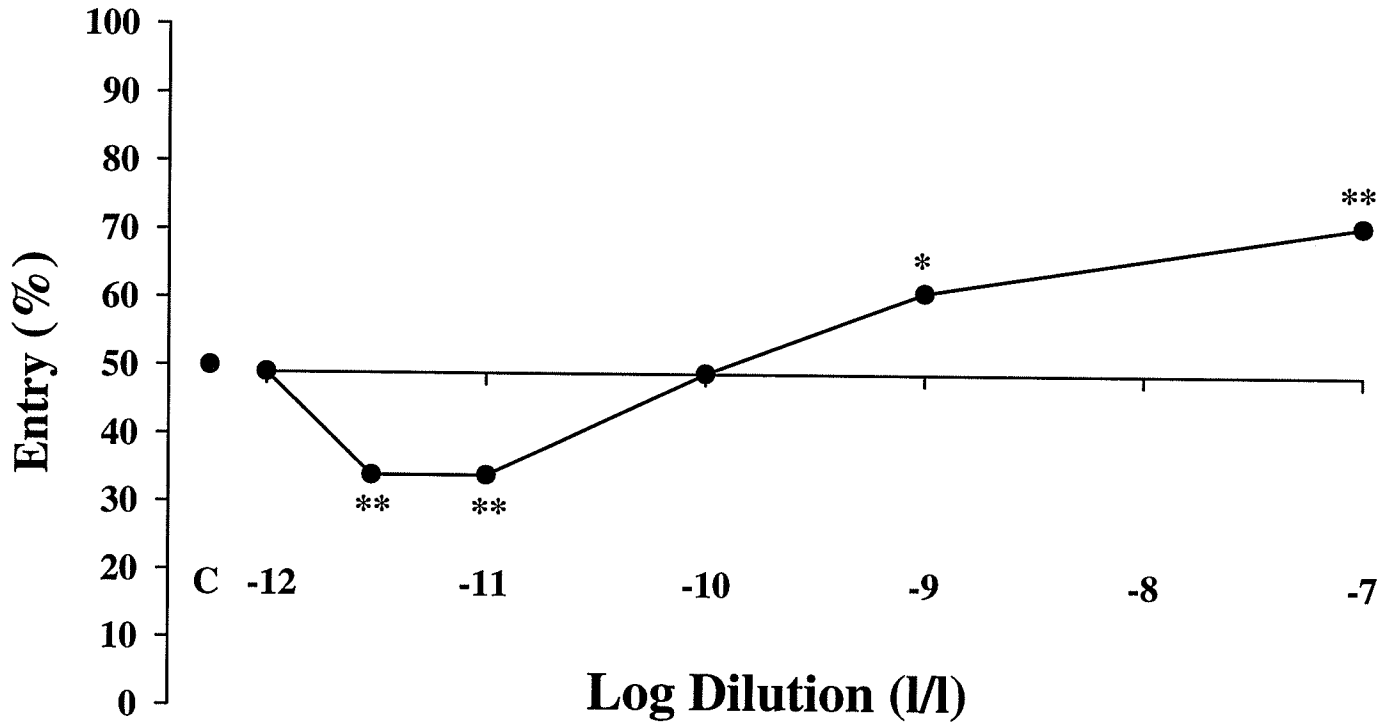


Figure 4.4 Preference/avoidance reactions of lake char to conspecific bile at various dilutions. C=control response. (*) indicates significant departure from neutral reaction hypothesis ($P < 0.05$); (**) indicates departure from neutral reaction hypothesis at a highly significant level ($P < 0.01$).

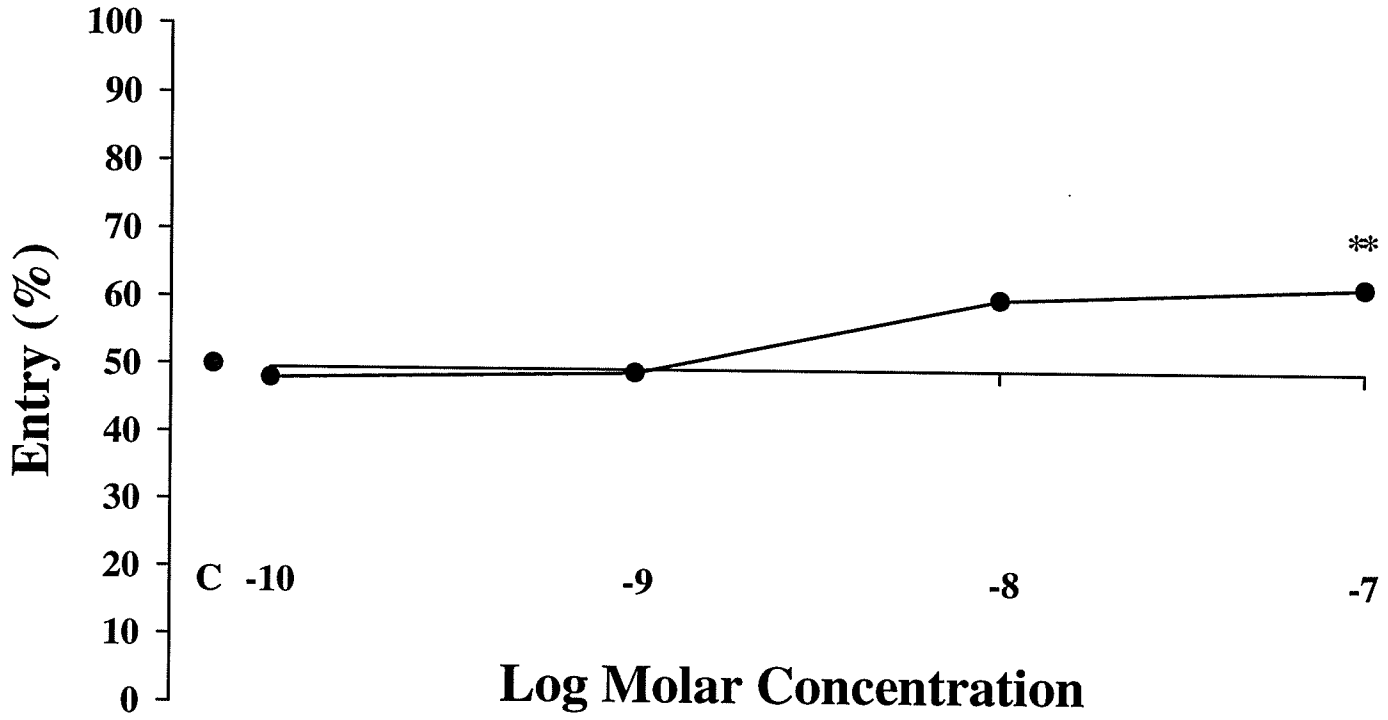


Figure 4.5 Preference/avoidance reactions of lake char to taurocholic acid at various concentrations. Labels are the same as Figure 4.4.

the influential role of natural excretions in mediating fish behaviour. Behaviour reactions to TCA and to bile acid related substances suggest bile acids are biologically important cues to fish.

Electroolfactogram recordings indicate that bile is an extremely potent olfactory stimulant. The majority of olfactory stimulatory components in bile is bile acids. Most of these components interact with amidated bile acid receptors in the olfactory epithelium. This evidence is consistent with chromatographic studies in which taurine amidated CA and/or CD are the major bile acids in salmonid bile (Sasaki 1966, Haslewood 1967a, Ripatti and Sidorov 1973; Denton et al. 1974). Quantitative evaluation of juvenile lake char bile showed that it contained about $3x10^2$ nmol/μl bile acids (Chapter 5). This explains why bile at 1-5 nl/l dilution is stimulatory, for olfactory thresholds to these bile acids are at sub-nanomolar concentrations. Besides bile acids, bile may contains a fair amount of free amino acids because olfactory responses to 100 nl/l bile were considerably reduced during adaptation to 10^{-3} M CYS.

Olfactory response thresholds for urine do not coincide with the amount of bile acids in the urine (Chapter 5). Thresholds would be 1-2 logarithmic units higher than the results observed if fish only respond to bile acids in the urine. If the amounts of urinary free amino acids in lake char are similar to the reports in carp and channel catfish (Ogata et al. 1983), the overall effects of the listed amino acids are, at best, at the same stimulatory range as the bile acids (Hara et al. 1993). Stimulatory effects of other nitrogenous end products, such as creatine, creatinine, uric acid, urea and NH_3, should be considered (Watts and Watts 1974). Creatine is a principal nitrogenous

should be considered (Watts and Watts 1974). Creatine is a principal nitrogenous constituent in urine for many species examined, especially in fasted fish (Smith 1929). Its amount sometimes exceeds 60% of total nitrogenous substances in the urine. Interestingly, creatinine at 10^{-8} M elicits detectable EOG responses in rainbow trout (Hara and Zhang, unpublished). When olfactory organs were stimulated by 0.1 M NH_4^+, strong olfactory tract discharges were observed, although the detection threshold was not determined (Goh and Tamura 1978). Other unknown biologically active components in urine may also contribute to the observed olfactory responses (Yano and Ishio 1978a, b, c; Colombo et al. 1982, Liley 1982; Yamazaki 1990; Moore and Scott 1992; Scott and Liley 1994; Scott et al. 1994).

A good correlation has been observed between behaviour preference reactions and physiological responses in terms of thresholds and response intensity in the present study (Fig. 4.2-5; Chapter 2). At the physiological detection range, fish have shown preference behaviour to all three types of stimuli. The results are consistent with behaviour studies in Atlantic salmon and Arctic char using bile, intestinal contents or urine as stimuli (Selset and Døving 1980; Olsén 1987; Stabell 1987; Moore et al. 1994). Aggressive behaviour observed during the urine stimulation was similar to the reaction of fighting for food. A similar observation termed feeding-territory-defence behaviour has been reported in Atlantic salmon parr in response to the urine stimulation (Moore et al. 1994).

The reason for biphasic behaviour responses to bile remains to be determined. The fact that lake char are indifferent to TCA stimulation at low concentration (Fig.

4.5) casts doubts that avoidance behaviour to low concentration bile is due to bile acid components. In some cases, however, behaviour reactions to mixture components are unpredictable on the basis of that to individual components in the mixture (Hashimoto et al. 1968; Adron and Mackie 1978; Honda 1980; van den Hurk and Lambert 1983; Saglio et al. 1990). Biphasic behavioural responses to seminal fluids in the black bullhead *Ictalurus melas* were observed (Rubec and Thomas 1979). Ovulated female African catfish prefer to low concentrations of seminal vesicle fluid of conspecific male, but avoid to that of high concentration. Some amino acids and bile acids induce biphasic behaviour responses in European glass eels (Sola et al. 1993; Sola and Tosi 1993). A few bile acids become repulsive at high concentration (Sola and Tosi 1993).

Behavioural thresholds for chemical cues are 2-4 logarithmic units lower than olfactory or gustatory thresholds examined electrophysiologically in Arctic char (Jones and Hara 1985, Sveinsson 1985; Sveinsson and Hara 1990a, Hara et al. 1993; Sveinsson, per communication). Similarly, lake char showed avoidance behaviour to bile at concentrations below olfactory thresholds. It is unknown whether this is a result of technical limitation of electrophysiological detection or the involvement of other chemosensory organs. Data are lacking as to how the central nervous system integrates the information from several chemosensory organs. Nevertheless, involvement of the gustatory system in bile acid detection should be considered. The gustatory sensitivity to bile acids in salmonids, unlike that to amino acids (Sutterlin and Sutterlin 1970; Mauri et al. 1983; Hara et al.1993, 1994), is equivalent or even higher than the olfactory sensitivity (Hara et al. 1984; Zhang and Hara 1991; Hara

and Zhang, unpublished). The specificity of gustatory responses to bile acids are clearly different from that of olfactory responses described in Chapter 2. Bile acids other than TCA in bile could have been detected by gustation or olfaction, or both, which induces avoidance behaviour. Furthermore, the gustatory system is highly sensitive to some bio-toxic substances such as tetrodotoxin and saxitoxin (Yamamori et al. 1988). It has been indicated that fish bile contains some unknown toxic bio-active substances (e. g. Chen et al. 1991; Wachtmeister et al. 1991). It may be possible that some toxicants in bile are detected through gustation and the avoidance behaviour becomes evident when, at low concentrations, the attractant information is outweighed by warning signals.

In summary, bile, urine and authentic bile acid TCA are effective olfactory stimulants and are able to elicit behaviour reactions in lake char. To some extent, lake char behaviour responses are stimulus-specific. Since natural biological excretions are comprised of many unknown substances, an approach towards characterization of biological substances in natural excretions would shed light on our understanding of the mediative role of chemical signals in fish behaviour. Therefore, I decided to examine the chemical composition of bile acids produced and released by lake char, which is detailed in the following chapter.

CHAPTER 5

HPLC Analyses of Bile Acids in Lake Char

INTRODUCTION

In previous chapters, I have shown that the lake char olfactory system is highly sensitive to bile acids. The sensory detection of bile acids is mediated by specific receptors (Chapter 2, 3). Also, the perception of water scented with bile acid substances triggers behavioural reactions in lake char (Chapter 4). Thus, the kinds of bile acids that fish actually released into water become an important issue if bile acids function as chemcial signals in mediating fish behaviour. Using electrophysiological cross-adaptation, Quinn and Hara (1986) showed that olfactory responses to tank water that held conspecific coho salmon were reduced to half during adaptation to 10^{-5} M TCA. However, there is little information regarding the bile acid excretion in fish. Knowledge on the nature of bile acids in fish is mainly restricted to biliary bile acids.

Sulphated bile alcohol, mainly 5α-cyprinol and 5α-chimaerol, and C_{24} bile acids, mainly CA, CD, DC and hesmulcholic acid, are the principal bile acids in bile in over 70 teleost species examined (ref. Haslewood 1967a, 1978; Tammer 1974). The C_{24} bile acids are taurine amidated and/or sulphated, although rainbow trout bile may have glycine amidated bile acids (Denton et al. 1974). Recently, cysteinolic acid amidated bile acids are found in the red seabream *Pagrosomus major* (Une et al. 1991). The formation of cysteinolic acid amidated bile acids appears related to dietary

contents of cysteinolic acid. A variety of biliary bile acids in fish may lead to different bile acid end products in the water.

Sea lamprey larvae, ammocoetes, produce a species-specific bile acid, petromyzonol sulphate (Haslewood and Tokes 1969; Li et al. 1995). However, its degraded compound, petromyzonol, constitutes the largest portion of bile acids in the larvae holding water (Li et al. 1995). In rainbow trout, bile acids in the faeces are similar to those in the bile (Sacquet et al. 1979). In mammals, it has been established that bile acids undergo considerable catabolic reactions in the intestine (ref. Hayakawa 1973; Macdonald et al. 1983; Ostrow 1993). Clearly, a systematic study on production of bile acids and their metabolite profile of excretion is needed to understand the chemical identity of bile acids that excreted by fish in the water.

My investigation is aimed to determine the composition of bile acids produced and released by lake char. Ion-pairing HPLC, combined with immobilized enzymatic post-column reactions, was used for bile acid analysis. Several analytical methods, including deamidation, sulphate solvolysis, ketonic reduction and hydrolysis of glucuronides were applied for detection and identification of bile acids in biological samples. By examining bile acids in bile, faeces, urine and tank water from both juvenile and adults, I demonstrate that the bile acids released by fish are among the most stimulatory for fish olfaction. These bile acids could be discriminated from one another by specific olfactory receptors. The release of characteristic bile acids in prespawning fish suggests that bile acids may function as chemical signals during spawning.

MATERIALS AND METHODS

Experimental animals

Lake char were raised and maintained as described in Chapter 2. Juveniles were 2-4 yrs of age and sexually quiescent. The gender and the stage in the reproduction cycle of adults were examined after sacrifice. Faeces were collected from females 2-3 months before spawning. Other four adults used in the experiments were 1) prespawning female ($♀_1$), 1-2 weeks before spawning, 2) spawning female ($♀_2$), 1-2 days before spawning, 3) mature male ($♂_1$), about 4 weeks before spawning, and 4) prespawning male ($♂_2$), 1-2 weeks before spawning.

Solvents and reagents

Reference bile acids and [^{3}H]TCA were from the same source as in Chapter 2 and 3. Solvents and other reagents were purchased from Sigma Chemical or Fisher Scientific at HPLC grade or the purest form available. Deionized water (DDW) purified by a MilliQ water system (Millipore, MA. USA) was used in the experiments.

Sample preparation and partial purification

Bile

Bile was collected from 3-day fasted juveniles (total length 25-31 cm) and 5-day fasted adults. Each fish was killed with a high dose of MS222 and a blow to

the head. The bile was obtained from the gall bladder with a disposable syringe. The bile volume was measured and diluted about 2 times DDW before extraction.

Reversed-phase C_{18} bonded-phase cartridges (Spe-ed, Applied Separations, PA, USA) were used for partial purification. Cartridges were activated with 5 ml methanol, 5 ml DDW-methanol (1:1), followed by 5 ml DDW before sample loadings. Approximately 9 pmol of [^{3}H]TCA (sp. act. 2.0 Ci/mmol) was added to each sample for recovery estimation. Bile acid components loaded in cartridges were eluted with 4 ml methanol after a wash with 3-ml DDW. Elution was by gravity. The extract from each sample was concentrated to 2 ml in methanol and the recovery was examined. Extracts were kept at -100°C until bile acid analysis.

Faeces

Fish faeces (intestinal contents) were collected from lower portion of the fish intestinal tract (within 5 cm distance from the anus) 3 days after last feeding. Each specimen was freeze-dried and divided into two. One was added with two reference bile acids, ursodeoxycholic acid (UC) and DC (0.4 μmol), to examine the recovery. Similar amounts of trout food pellets were extracted at the same time to examine whether fish food contains bile acids. All samples were also added [^{3}H]TCA to follow bile acid losses during the extraction. With minor modification, the method of Locket and Gallaher (1989) was used for bile acid extraction from faeces. Briefly, 2 ml of absolute ethanol was added to each of screw capped tube containing about 100 mg faeces. Tubes were tightly capped and sonicated in a bath sonicator for 30 min. The

tubes were then placed in a heating block (80-100°C) and refluxed for 25 min. After refluxing, each sample was centrifuged at 1500 x g for 10 min, and the supernatant was transferred to another tube. The sample was resuspended in 2 ml 80% ethanol, refluxed and centrifuged as before. The second supernatant was pooled with the first one. The same procedure was repeated two more times with 2 ml dichloromethane-methanol (1:1, v:v). The combined supernatant were evaporated to dryness with nitrogen gas streams at a temperature below 35°C. The dried residues were resuspended with 0.6 ml methanol, vortexed to solubilize bile acids, and finally diluted to 4 ml with DDW before being loaded onto C_{18} cartridges for partial purification.

Urine

The method described by Parry et al. (1994) was used for urine collection. Each fish was cannulated by a flexible silicone tubing that fitted the urinary orifice. The catheter was connected to a PE 190 or PE 240 Intramedic tubing (Clay Adams, NJ, USA) and positioned over a collecting beaker. The catheterized fish was held individually in a plexiglass box. Plain water (11-12°C) entered the anterior end and overflowed to waste from the posterior end of the box. The urine collection was started 9 hrs after catheterization to minimize possible side effects due to disturbance and MS222 anaesthetization (Hunn and Willford 1970; ref. Hunn 1982). Bile acids in urine were extracted using C_{18} cartridges.

Tank water

Water samples were collected from tanks holding 3-day fasted juveniles or 5-day fasted adults. Adults were held individually in a fibreglass tank (150 litres water) at a water flow of 1 l/min. Thirteen juveniles with total body weight equivalent to the adults were kept in one tank at the same water flow rate as for the adults. Water samples were siphoned from the surface of tank water and passed through a glass microanalysis filter, by which a stainless-steel porous matrix (100 mesh) filtrated out large particles. Bile acids in the water were extracted by passing 8 litres of water through four of C_{18} cartridges in 9-12 hrs. The same amount of plain water with or without reference bile acids was treated in a similar manner to serve as controls.

Analytical methods

Hydrolysis of taurine or glycine amidates

N-acyl conjugated bile acids were subjected to enzymatic hydrolysis with choloylglycine hydrolase (EC 3.5.1.24) in acetate buffer (0.1 M sodium acetate buffer, pH 5.6) and incubated overnight at 37°C. After cooling to room temperature, samples were purified using C_{18} cartridges similar to those previously described, except for washing the loaded column with an additional 4 ml DDW before the bile acid elution.

Solvolysis of sulphate esters

The procedure described by Princen et al. (1990) was used for the solvolysis to free 3α-hydroxyl of the bile acids. Samples to be tested were dissolved in 120 μl DDW and sonicated for 5 min. Then, 4 ml of 1,4-dioxane containing 1 mM HCl was added to each sample. After incubated at 37°C for 16 hrs, the samples were dried under reduced pressure. Parallel control tests using TLS and DCS suggested that this procedure completely solvolized bile acid sulphates.

Ketonic reduction

A small portion of each sample was subjected to 3-oxo bile acid reduction before HPLC analysis to examine the content of ketonic bile acids. The procedure of reduction was the reverse reaction of 3α-hydroxyl oxidation (Tomkins 1956). Samples were incubated in 0.05 M KH_2PO_4 buffer (pH 7.0) containing 1.25 mg/ml of NADH and 2 units/ml 3α-hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.50, chromatographically purified) at 35°C for 20 min. These samples were then purified with C_{18} cartridges. Reference bile acid ODA (7α,12α-dihydroxy-3-oxo-5β-cholan-24 oic acid) was processed at the same time as a control.

Hydrolysis of glucuronides

Control tests using 3α,17β-dihydroxy-5α-androstane 17β-glucuronide (AG) showed that this steroid glucuronide was completely retained in the C_{18} cartridge under a protracted wash of DDW (60 ml). Thus, bile acid glucuronides in various extracts

were examined. β-Glucuronidase (1000 Sigma units, EC 3.2.1.31, Bacterial Type VII) in 0.05 M phosphate buffer (pH 6.8) was added to each dried sample that was sufficient for one trial of HPLC analysis. Hydrolysis of glucuronides was performed in a shaking water bath at 37°C for 24 hrs. After cooling to room temperature, samples were purified using C_{18} cartridges. A control sample containing AG, TCA, TCD, CD and DCS was processed similarly at the same time. Analysis of the control sample showed that 20 nmol of 3α,17β-dihydroxy-5α-androstane was completely liberated from AG by β-glucuronidase. The process had no effects on sulphate conjugation.

Group separation by LH-20

Sulphated (and glucuronide conjugates, if any) and nonsulphated bile acids can be separated from each other using liquid chromatography LH-20. Two grams of Sephadex LH-20 (Pharmacia LKB, Uppsala, Sweden), equilibrated overnight in chloroform-methanol (3:2, v:v) solvent saturated with NaCl, was packed in a 160 mm x 8 mm I.D. cartridge. Free and conjugated bile acids were eluted with 30 ml of the above solvent and sulphated bile acids were eluted with 10 ml methanol. Preliminary experiments indicated that TCA, TDC and CD were washed off by the first 20-ml chloroform-methanol solvent and TLS by the first 4-ml of methanol.

Apparatus and analytical conditions of HPLC

The HPLC system used consisted of 2 Model 302 solvent pumps, a Model 306

solvent pump, a Model 231-401 auto-sampling injector, a Model 704 system controller, a Model 620 data module, and a Model 201 fraction collector (Gilson Medical Electronics, WI, USA). A temperature control system (Waters, MA, USA) was used to keep the column at the constant temperature. A Shimadzu RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) was set at excitation 365 nm and emission 465 nm (Fig.5.1).

For HPLC analysis, 1 nmol of internal standard LC (or UC) was added to each sample, and dried under reduced pressure. Each sample was then dissolved in 100 μl of mobile phase solvent at the starting gradient. Twenty microlitres of the aliquot were injected into the 5-micron econosphere C_{18} column (150 mm x 4.6 mm I.D., Alltech, IL, USA). A Hamilton PRP-1 column (5 μm, 150 mm x 4.1 mm I.D., Hamilton, NV, USA) was also used for bile acid identification.

Individual bile acids were eluted by reversed-phase HPLC with mobile phase ammonium carbonate (0.3%, pH 8.2) and acetonitrile at a flow of 0.5 ml/min (26°C). The gradient started at 22% of acetonitrile and linearly increased to 24% at 40 min, 27% at 60 min, and 40% at 95 min. At 124 min, the acetonitrile concentration returned to 22% in 1 min and maintained until the next run (Fig. 5.2).

The bile acids separated on the reversed-phase column reacted online with β-NAD buffer in a custom-made post-column (20 mm x 4 mm I.D., Alltech) containing 3α-HSD that was mounted on glutaraldehyde-treated aminopropyl glass beads (75 Å pore size) (Marshell 1973). The β-NAD buffer was made up with 0.1 M Tris-HCl (pH 8.5), 1.7 mM EDTA, 0.8 M dithiothreitol and 38 μM β-NAD (Gallaher and

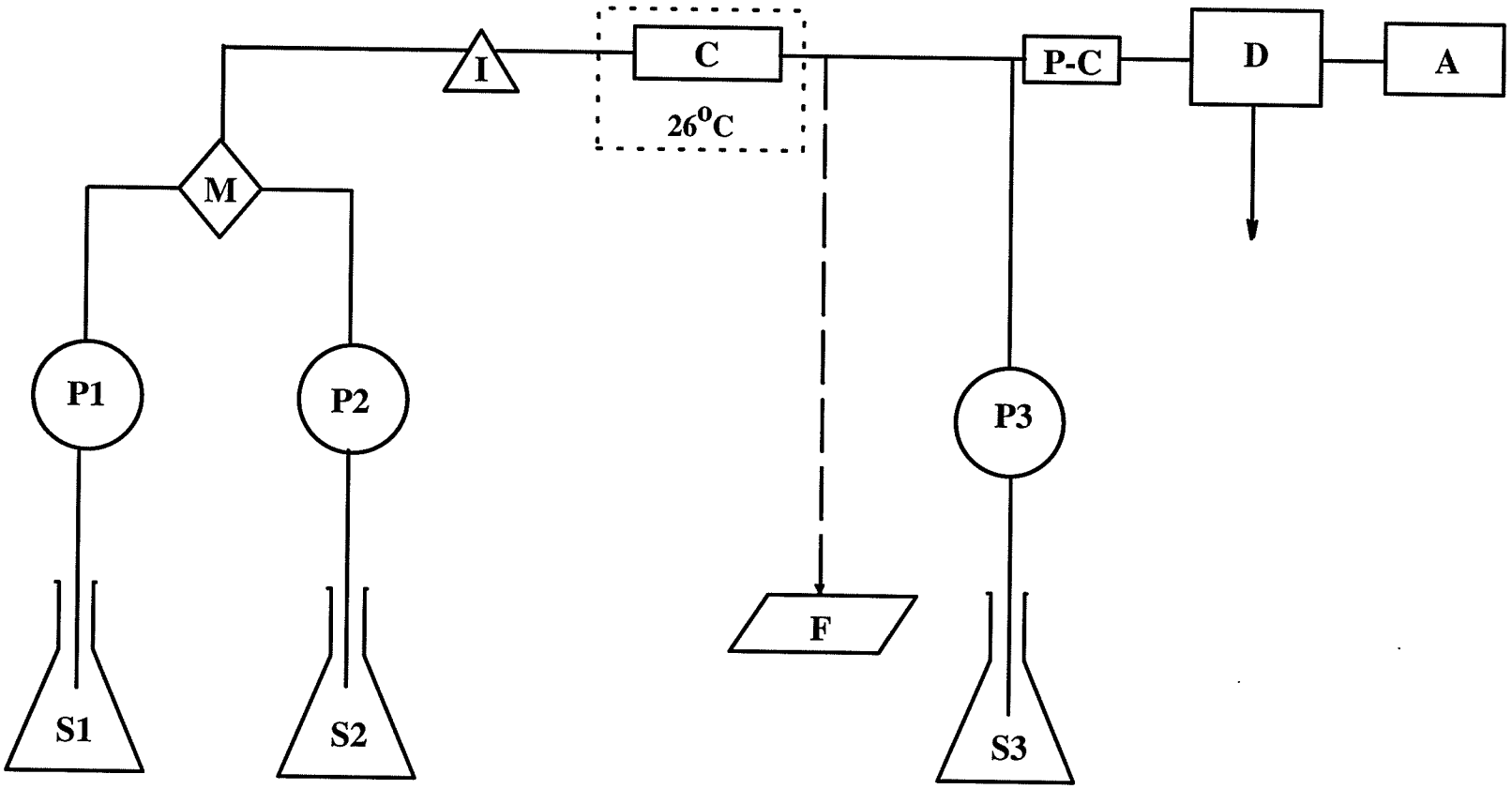


Figure 5.1 A diagrammatic illustration of the HPLC system. A=data analysis module; C=column; D=detector; F=fraction collector; I=auto-sampling injector; M=dynamic mixer; P1 to P3=pumps; P-C= post-column; S1 to S3=solvents. The dashed line around the column indicates a column oven.

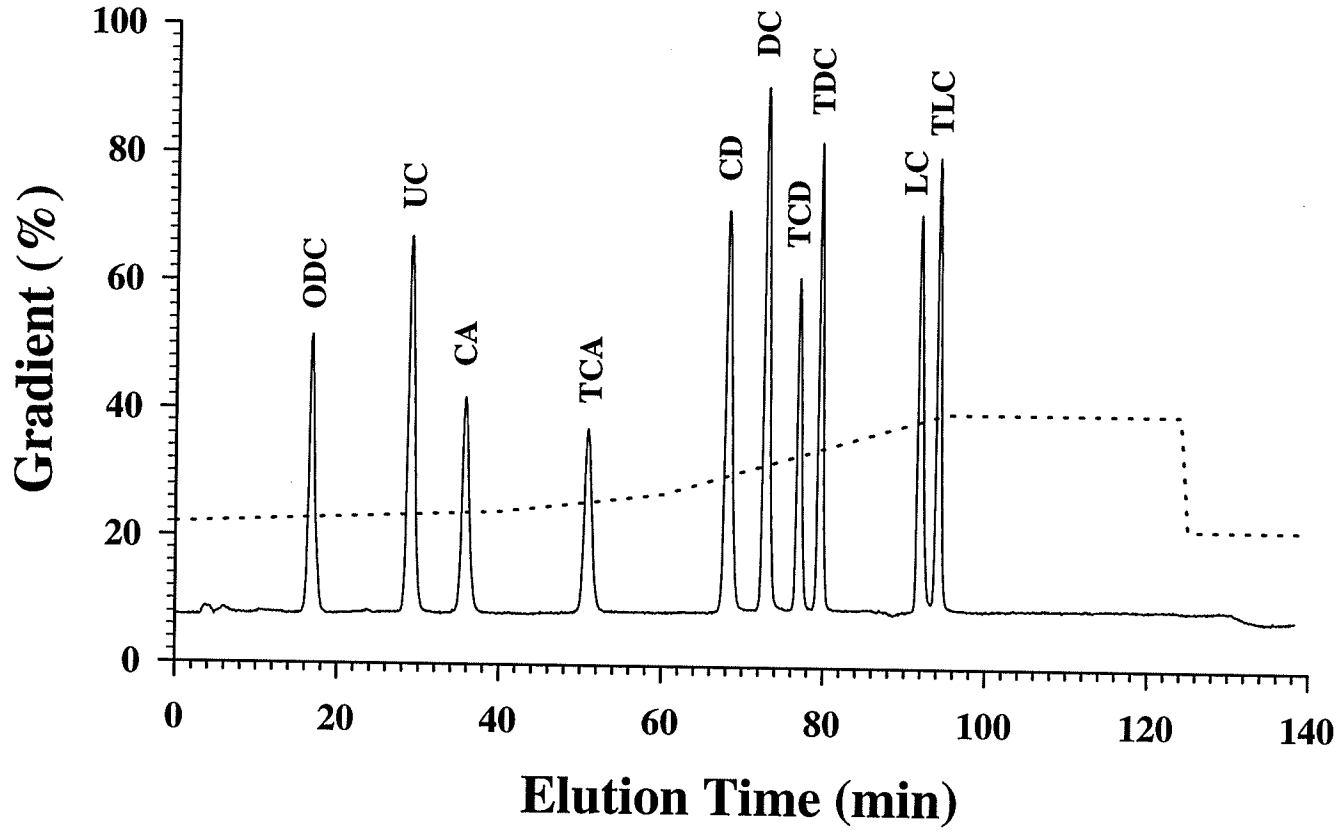


Figure 5.2 Baseline separation of 10 reference bile acids. ODC=7-oxodeoxycholic acid; UC=ursodeoxycholic acid; CA=cholic acid; TCA=taurocholic acid; CD=chenodeoxycholic acid; DC=deoxycholic acid; TCD=taurochenodeoxycholic acid; TDC=taurodeoxycholic acid; LC=lithocholic acid; TLC=taurolithocholic acid. Changes in elution gradients are shown as the dot line.

Franz 1990). The solution was introduced by means of a tee between the first and second columns (Fig. 5.1). The β-NADH generated by enzymic reactions was detected fluorometrically (Okuyama et al. 1978; Baba et al. 1980). The quantity of bile acids was determined by measuring the peak area of each bile acid as compared to that of the internal standard and calculated based on the calibration curve. The amount of an unidentified 3α-hydroxysteroid sulphate (UIS) was calculated using a calibration curve based on the internal standard. Blank tests were conducted for every type of extract in the absence of β-NAD in the buffer.

Electrophysiological responses to fractions

Olfactory responses to the above biological extracts were examined using EOG recording as described in Chapter 2. Extracts of juvenile bile, faeces, urine and tank water with approximately equipotence for EOG responses were injected into the column and eluates were collected before they reacted with β-NAD in the post-column (Fig. 5.1). The eluates were diluted with 4 times DDW and loaded onto C_{18} cartridges, because preliminary experiments indicated that, under this dilution, bile acid components would remain in cartridges during the loading. After washing the loaded cartridge with 5 ml DDW, the components were eluted by 4 ml methanol. Each fraction was then dried and resuspended in 0.5 ml methanol. An aliquot (10 μl) of suspension added to 10 ml plain water formulated the olfactory stimulant. Similar amount of the mobile phase solvent was processed in an identical manner as the fractions to serve as control (MF) and tested for EOG responses. Only background

responses were obtained during the MF stimulation. The experiments were conducted in duplicate from two individual extracts of the same type. Examination was repeated if the results from the duplicate were not consistent.

RESULTS

Recovery

Average recoveries of bile acids in bile, faeces, urine and tank water during extraction and partial purification using C_{18} cartridges are listed in Table 5.1. The overall recovery was over 90%. A value of 92.5±2.0% was obtained when known quantities of reference bile acids were added in crude faeces for recovery estimation. Analysis of control samples containing known amount of reference bile acids suggested that recoveries for various bile acids were similar during the extraction (above 90%).

Table 5.2 shows recoveries during the process of deamidation, sulphate solvolysis, ketonic reduction and hydrolysis of glucuronides. The results between reference bile acids and the [^{3}H]TCA tracings were in excellent agreement, except for the sulphate solvolysis, where the unlabelled tracers showed lower recoveries than [^{3}H]TCA during sulphate solvolysis.

Chromatographic separation

The chromatographic conditions were examined to obtain complete separation of free bile acids and their amidates. After preliminary experiments, a gradient

Table 5.1 Recoveries (as percentages of added [^{3}H]taurocholic acid) of bile acid extraction from various biological samples.

	Bile	Faeces	Urine	Water
Mean±SEM(%)	93.6±5.0	93.5±2.7	97.4±3.5	98.1±3.1

Table 5.2 Recoveries of bile acids (as percentages of added tracers) during the hydrolysis of amidates (d-amidation), sulphate solvolysis (d-sulphation), ketonic reduction (d-oxo) and hydrolysis of glucuronides (d-gluc).

Type of tracer[a]	d-amidation	d-sulphation	d-oxo	d-gluc
Labelled	99.7±9.9	98.4±7.1	97.6±15.4	-[b]
Unlabelled	90.8±5.0	71.5±3.4	96.2±1.9	102.4±2.38

[a] Labelled=[^{3}H]taurocholic acid; Unlabelled=reference bile acids.

[b] - =not examined.

elution mode using mobile phase acetonitrile-ammonium carbonate as described (Materials and Methods) was selected. A complete baseline separation to a mixture of 10 free and taurine amidated reference bile acids was achieved (Fig. 5.2). All bile acids were eluted within 95 min. The elution time for glycine amidated bile acids were determined in a separate run. Bile acids were eluted in a definite order depending on the number, position and configuration of hydroxyls on the steroid nucleus. Free bile acids were eluted earlier than their respective glycine amidates, and the latter was washed off earlier than the corresponding taurine amidates (Table 5.3). The reliability of elution time for individual reference bile acids was excellent ($CV < 1\%$, n=2).

Figure 5.3 shows calibration graphs for 10 reference bile acids. A linear relationship between the peak area and the amount of bile acids injected was obtained. Correlation coefficients for the linear regression ranged from 0.98 to 0.99 (n=12 for the free and n=8 for the amidated bile acids). Detection limits were approximately 10 pmol, depending on the efficiency of the detector and the 3α-HSD activity in the post-column. Quantitative bile acid analysis showed high reproducibility ($CV < 6.5\%$ for all reference bile acids, n=2).

Bile acid identification

The major bile acids identified and their presence in the extracts of bile, faeces, urine and holding water from lake char are listed in Table 5.4. The bile acid identification was done by the following means: 1) comparing the relative capacity

Table 5.3 Relative capacity ratios of reference bile acids for the econosphere C_{18} column (outside parentheses) and Hamilton PRP-1 column (inside parentheses). Values are relative to the retention time of lithocholic acid.

Bile acids	Free acids	Taurine	Glycine
7-Oxodeoxycholic acid	0.156 (0.116)	- [a]	-
Ursodeoxycholic acid	0.292 (0.225)	-	-
Cholic acid	0.374 (0.247)	0.540 (0.382)	0.475 (0.309)
Chenodeoxycholic acid	0.735 (0.579)	0.837 (0.777)	0.798 (0.671)
Deoxycholic acid	0.788 (0.671)	0.866 (0.835)	0.838 (0.754)
Lithocholic acid	1.000 (1.000)	1.028 (1.057)	1.001 (1.005)

[a] - = not examined.

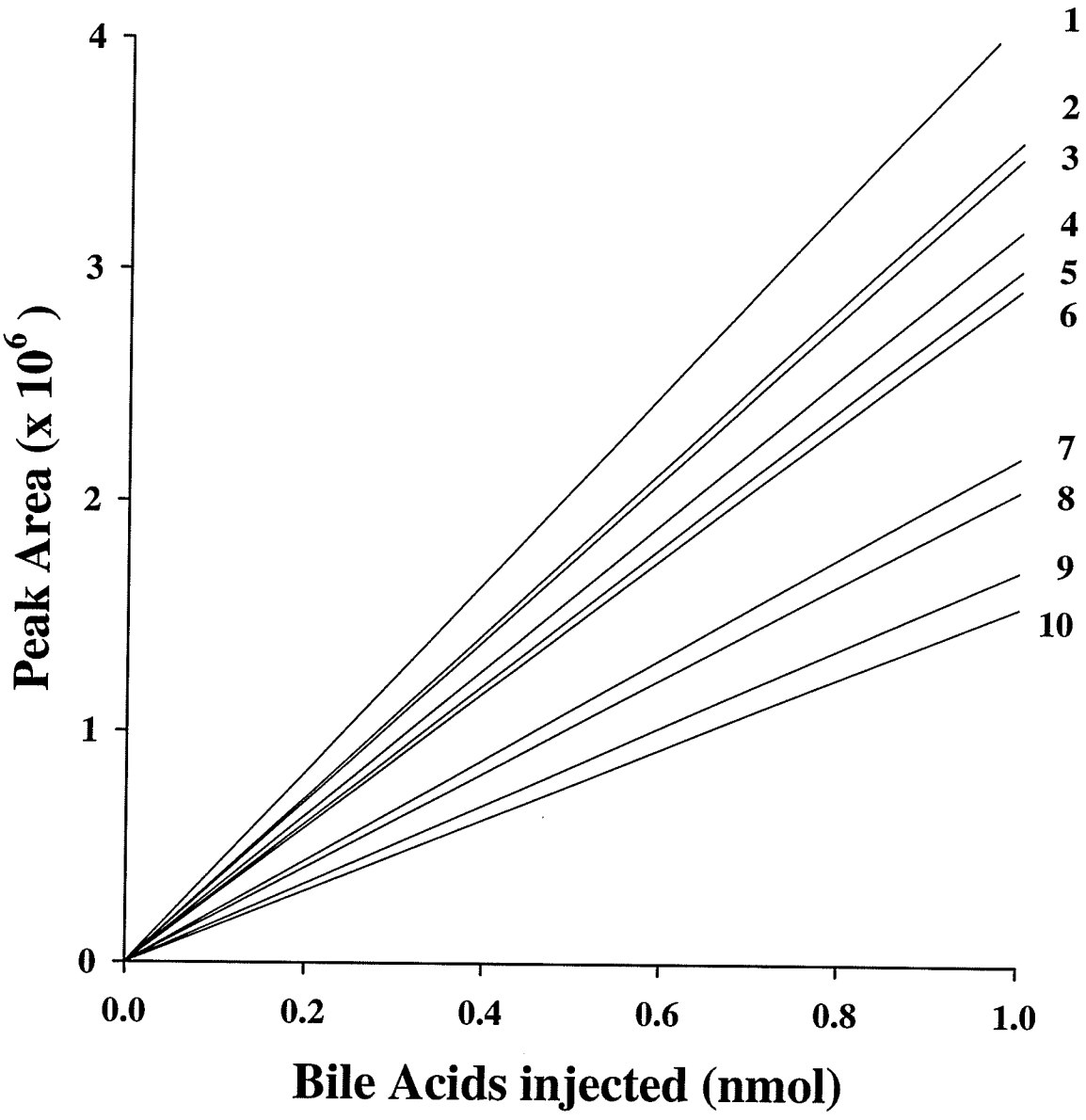


Figure 5.3 Calibration graphs for reference bile acids. Data were constructed from chromatograms obtained by injecting mixtures of reference bile acids. Numbers from (1) to (10) in order refer to deoxycholic acid, ursodeoxycholic acid, chenodeoxycholic, lithocholic acid, taurolithocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, 7-oxodeoxycholic acid, cholic acid and taurocholic acid, respectively.

Table 5.4 Bile acid composition.

Source	Compounds[a]									Total[c]
	TCA	TCD	TLC	UIS[b]	3α-S	CA	CD	TOCN	β-Gluc	
In Bile (nmol/ul)										
Juveniles	268.5±5.1[d]	15.0±0.8	0.1±0.1	1.5±0.1	7.8±0.2	N. D.	N. D.	20.5±0.0	N. D	293.0±4.8
Adults	84.3±7.2	22.9±4.4	0.1±0.0	1.6±0.5	9.6±1.4	N. D.	N. D.	-	-	118.6±7.6
In Faeces (nmol/mg)										
Juveniles	23.4±0.7	3.1±0.3	trace	trace	1.3±0.1	N. D.	N. D.	3.5±0.7	N. D.	27.9±0.5
Adults	26.1±1.4	9.6±0.9	trace	trace	2.6±0.6	N. D.	N. D.	-	-	38.7±2.0
In Urine (nmol/hr)										
Juveniles	2.6±2.4	1.4±1.4	N. D.	trace	0.2±0.2	N. D.	N. D.	-	N. D.	4.2±3.9
♀1	0.6±0.0	0.6±0.3	N. D.	0.1±0.0	N. D.	N. D.	N. D.	-	N. D.	1.3±0.4
♂1	22.2±1.8	3.2±1.2	N. D.	2.8±0.6	-	3.1±3.1	0.2±0.2	-	N. D.	31.4±0.9
♂2	42.4±11.1	30.4±12.9	N. D.	17.0±4.6	5.2±0.9	20.5±11.2	17.9±9.2	-	N. D.	133.4±29.5
In Water (nmol/min per fish)										
Juveniles	0.4±0.0	0.2±0.1	N. D.	trace	trace	N. D.	N. D.	N. D.	N. D.	0.5±0.1
♀1	102.9±55.0	104.2±51.2	N. D.	trace	-	N. D.	N. D.	-	-	207.1±103.5
♀2	7.4±5.9	4.9±3.0	N. D.	trace	0.6±0.6	N. D.	N. D.	-	N. D.	13.0±9.5
♂1	3.4±0.0	3.8±1.4	N. D.	trace	-	N. D.	N. D.	-	-	7.2±1.4
♂2	1.0	0.7	N. D.	0.3	-	N. D.	N. D.	-	-	2.0
Others										
Ovarian fluid	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.
Seminal fluid	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.
Controls										
DDW	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.
Dechlorinated water	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.
Food pellets for juveniles	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.
Food pellets for adults	N. D.	N. D.	N. D.	N. D.	-	N. D.	N. D.	-	-	N. D.

[a] UIS=unidentified compound; 3α-S=all sulphated bile acids detected after solvolysis. The major bile acid sulphate is taurine amidated 7-oxodeoxycholic acid. TOCN=taurine amidated 3-oxocholanic acid. β-Gluc=glucuronide conjugated bile acids. Refer the legend of Figure 5.2 for abbraviations of other compounds

[b] The quantity of UIS is arbitrarily calculated using the internal standard (lithocholic acid) calibration curve.

[c] The value of TOCN was not included in the total for purpose of comparison between juveniles and adults.

[d] The value is mean and standard error of samples form different individuals or from the same individuals collected at different time periods. N. D. =not detected. - =not examined. trace=detectable but the amount is below the significant digits as shown.

ratio (k') of peaks between the samples and the reference bile acids, 2) examining peak shapes of the samples spiked with the tentative authentic compounds, and after their deamidation, 3) repeating the above two procedures in another column of different physical behaviour, 4) changing mobile phase gradients and comparing the k' value of peaks between the samples and the reference bile acids again. Figure 5.4 shows chromatograms of bile from juvenile lake char and the profile changes after deamidation, sulphate solvolysis, solvolysis after deamidation and 3α-oxo reduction. Examination of deamidated extracts with or without reference bile acid ACA suggested that lake char produce and release 5β-, not 5α-, bile acids.

An unidentified compound (UIS, k' = 0.882) forms a large fraction of total bile acids in the prespawning male urine (see below) and traces in other extracts. The elution time for UIS was unchanged after deamidation. This compound has a sulphate ester group(s) located at a position(s) other than at 3α-hydroxyl, because UIS was detected before solvolysis and the solvolysis yielded a compound UI that is less polar than LC. These observations suggest that UIS is a nonamidated 3α-hydroxysteroid sulphate. This compound may be an uncommon bile acid sulphate or a steroid sulphate that could react with 3α-HSD. An attempt was made to find the tentative compound for UIS. However, none of the 3α-hydroxyl steroids listed in Table 5.5, which may interact with 3α-HSD, had similar physical properties as UIS.

Analysis of sulphate fractions from the LH-20 partial separation showed that all sulphated bile acids in extracts, other than UIS, had a sulphate ester on 3α-hydroxyl.

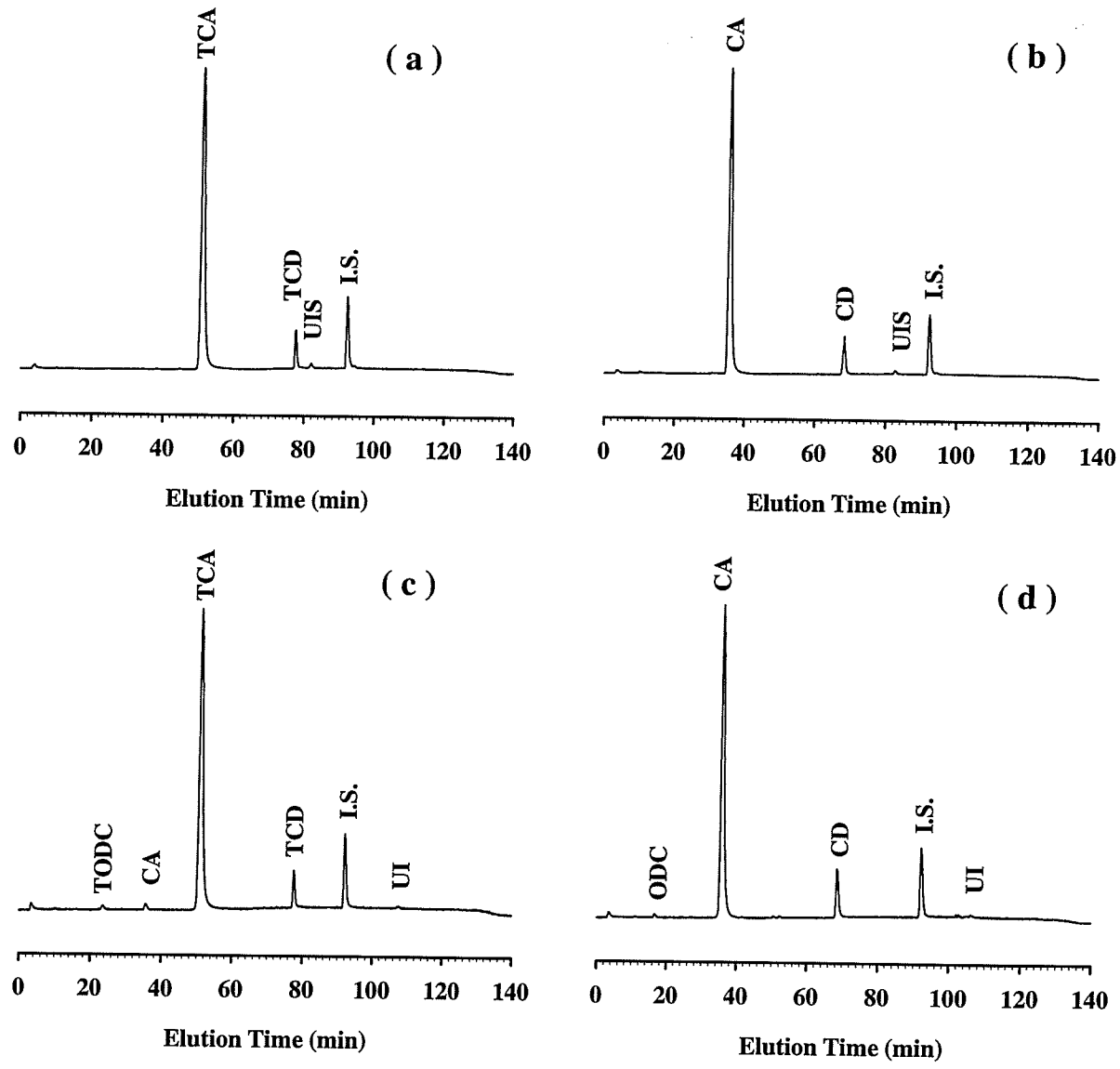


Figure 5.4 Examples of HPLC analysis profile: **(a)** juvenile bile; **(b)** after deamidation; **(c)** after sulphate solvolysis; **(d)** after solvolysis and deamidation; **(e)** after ketonic reduction. CA=cholic acid; CD=chenodeoxycholic acid; UIS=unidentified compound; UI=compound of UIS after solvolysis; ODC=7-oxodeoxycholic acid; I.S.=internal standard (lithocholic acid). Abbreviations having "T" indicate equivalent taurine amidates.

Table 5.5 List of compounds that were excluded as candidates for UIS.

Sulphates of
3α,7α,12α,24-Tetrahydroxy-5α-cholane
3α,7α,12α,24-Tetrahydroxy-5β-cholane
3α,17β-Dihydroxy-5α-androstane
3α,17α-Dihydroxy-5β-androstane
3α,17α,20α-Trihydroxy-5β-pregnane
3α,11β,17α,20α-Tetrahydroxy-5β-pregnane
3α,11β,21-Trihydorxy-5α-pregnane-20-one
3α,11β,21-Trihydorxy-5β-pregnane-20-one
3α,17β,21-Trihydroxy-5β-pregnane-20-one
3α,20α-Dihydroxy-5β-pregnane
3α,21α-Dihydroxy-5β-pregnane-20-one
3α,17α,21-Trihydroxy-5α-pregnane-11,20-dione
3α,17α-Dihydroxy-5α-pregnane-20-one
3α,17β-Dihydroxy-5β-estrane
Acetate conjugates of steroids (Do not react with 1,4-dioxane)
β-glucuronide conjugates of steroids (Do not react with 1,4-dioxane)
Cholestanes (Do not react with 3α-HSD)
Steroids with unsaturated bonds at A ring (Do not react with 3α-HSD)

Bile acid composition

Bile acids were not detected in plain water or in fish food pellets. They were also not detected in ovarian and seminal fluids of lake char (Table 5.4). In humans, urine contains higher concentrations of bile acid glucuronides than other sources (Almé and Sjövall 1980; Ostrow 1993). Therefore, the investigation of bile acid glucuronides focused on bile acid glucuronides in urine. As shown in Table 5.4, no bile acid glucuronides were detected in all extracts examined.

In bile

The bile acid components in bile collected from individual juveniles was identical. Bile contained an average of 3 x 10^2 nmol/μl of bile acids (Table 5.4). The composition of bile acids were consistent among individuals. The quantity of bile acids was directly correlated with the volume of bile (CV=4.2%, n=5). However, a large disparity was obtained among individuals if the data were further divided by fish body weight (CV=24.7%).

Taurine amidated CA and CD (TCA and TCD), made up over 90% of the total bile acids in bile. Taurolithocholic acid comprised less than 0.1% of the total. Compound UIS accounted for less than 1%. Chromatograms of samples after ketonic reduction process contained another mid-sized peak, TLC. This indicates the presence of approximately 7% taurine amidated 3-oxocholanic acid (TOCN). About 3% of bile acids were 3α-hydroxy sulphated. The major bile acid sulphate was tentatively identified as taurine amidated 7-oxodeoxycholic acid (TODC). Traces of CA and CD

might also be conjugated with sulphate esters.

The volume of bile, as well as the bile acid pool, increased in adult fish. However, bile acid concentrations in bile from adults was about two-fifth those of juveniles (Table 5.4). The profile of bile acid composition in bile of adults was similar to that of juveniles, except that the proportions of TCA and TCD (TCA/TCD ratio) was much lower in the bile from adults (Fig. 5.5).

In faeces

The intestine of juveniles fasted for 3-day was almost empty. Faeces collected from the last portion of the juvenile intestine contained 31.2±0.7 nmol/mg bile acids. Bile acid components in the faeces were similar to those in bile, containing over 80% of TCA and TCD, about 4% of TODC sulphate, 10% of TOCN, and traces of TLC and UIS (Table 5.4). The TCA/TCD ratio in the faeces was half of that in bile (Fig. 5.5).

As in bile, the major difference of bile acid composition in faeces between adults and juveniles was the TCA/TCD ratio; the former was about one-third of the latter (Fig.5.5). Also, the concentration of sulphated TODC was slightly higher in adults (7% vs 5%).

In urine

There was sexual dimorphism in urinary bile acid excretion (Table 5.4). Less than 2 nmol/hr of bile acids was present in the urine of mature female $♀_1$. This

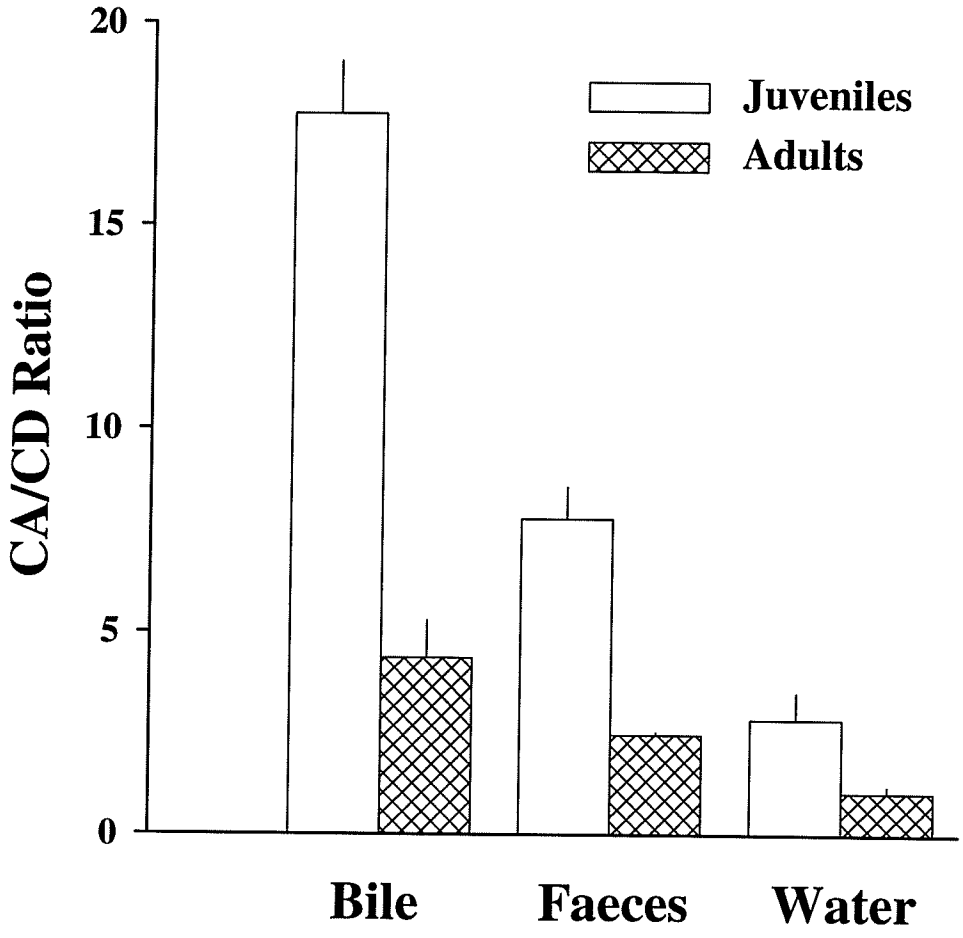


Figure 5.5 Differences in the proportion of taurocholic acid and taurochenodeoxycholic acid (TCA/TCD ratio) in bile, faeces and tank water of juveniles (open bars) and adults (crosshatched bars).

amount was even less than that in some juvenile urine (Table 5.4). Urinary bile acids in mature males was 20-100 times those in the female. Interestingly, the urinary bile acid concentration in a prespawning male $\sigma^{\prime}_2$ was 4-10 times more than that in $\sigma^{\prime}_1$. Urine of $\sigma^{\prime}_2$ contained a large quantity of UIS. Its peak area accounted for one-third of the total bile acids in the urine (Fig 5.6). Moreover, there were also substantial amounts of free bile acids, CA and CD, which comprised approximately one-fourth of total bile acids on average in the urine of $\sigma^{\prime}_2$. The composition of individual bile acids in $\sigma^{\prime}_2$ urine changed temporally as evidenced by three successive 12-hr-period collections (Fig. 5.7). An increase in urinary excretion of sulphated TODC in $\sigma^{\prime}_2$ was also observed, although its ratio over the total urinary bile acids decreased by almost one half as compared to the juvenile (3% *vs* 5%).

In tank water

Bile acids released into water may be detected by fish chemosensory systems. Analysis of water sampled on three consecutive days from the tank holding juveniles showed an average of 3 nmol/min per kilogram body weight (or 0.5 nmol/min per fish) of bile acids was released into the water (Table 5.4). A comparable figure was found in $\sigma^{\prime}_1$ tank water (2 nmol/min per kg). Water from $\sigma^{\prime}_2$ tank characteristically showed a higher concentration of UIS than the water from other tanks. Higher concentrations of bile acids were found in the tank water that held prespawning female φ_1 and spawning female φ_2 than in other tank water. Furthermore, the concentration varied up to 100 fold during three successive sampling days (Fig. 5.8). Bile acid

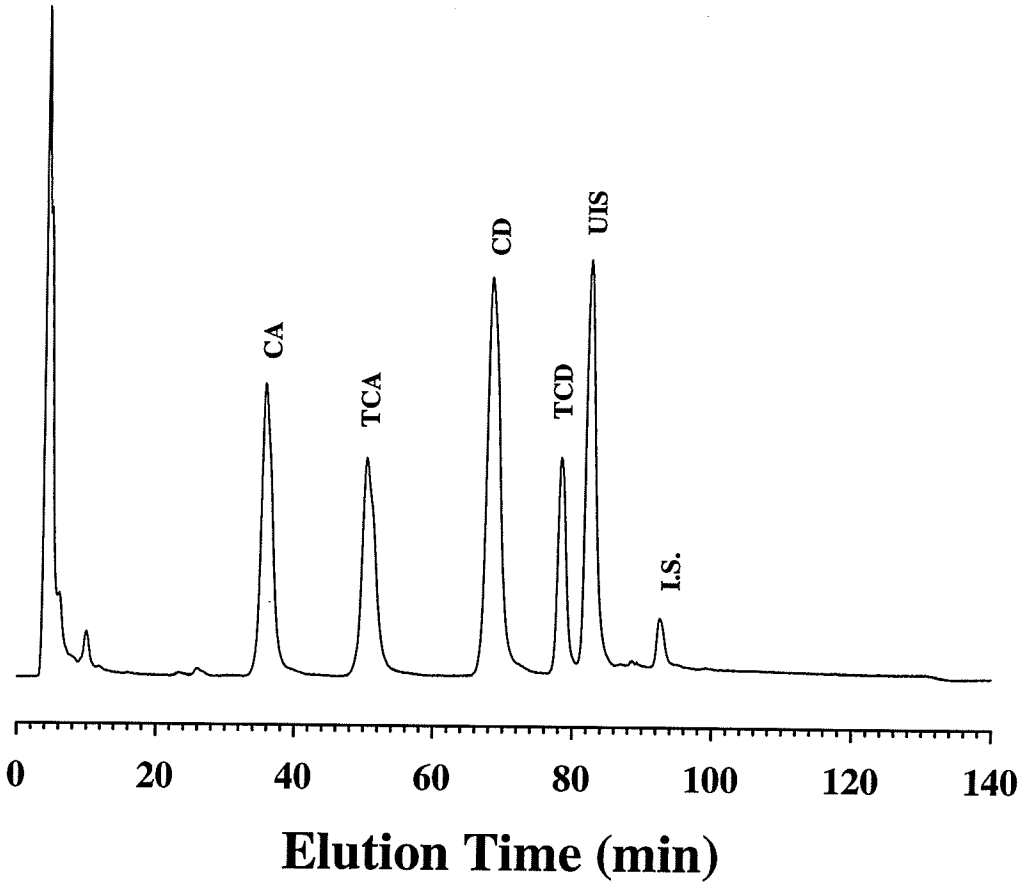


Figure 5.6 The chromatogram of bile acids in the urine of a prespawning male. CA=cholic acid; TCA=taurocholic acid; CD=chenodeoxycholic acid; TCD=taurochenodeoxycholic acid; UIS=unidentified compound; I.S.=internal standard (lithocholic acid).

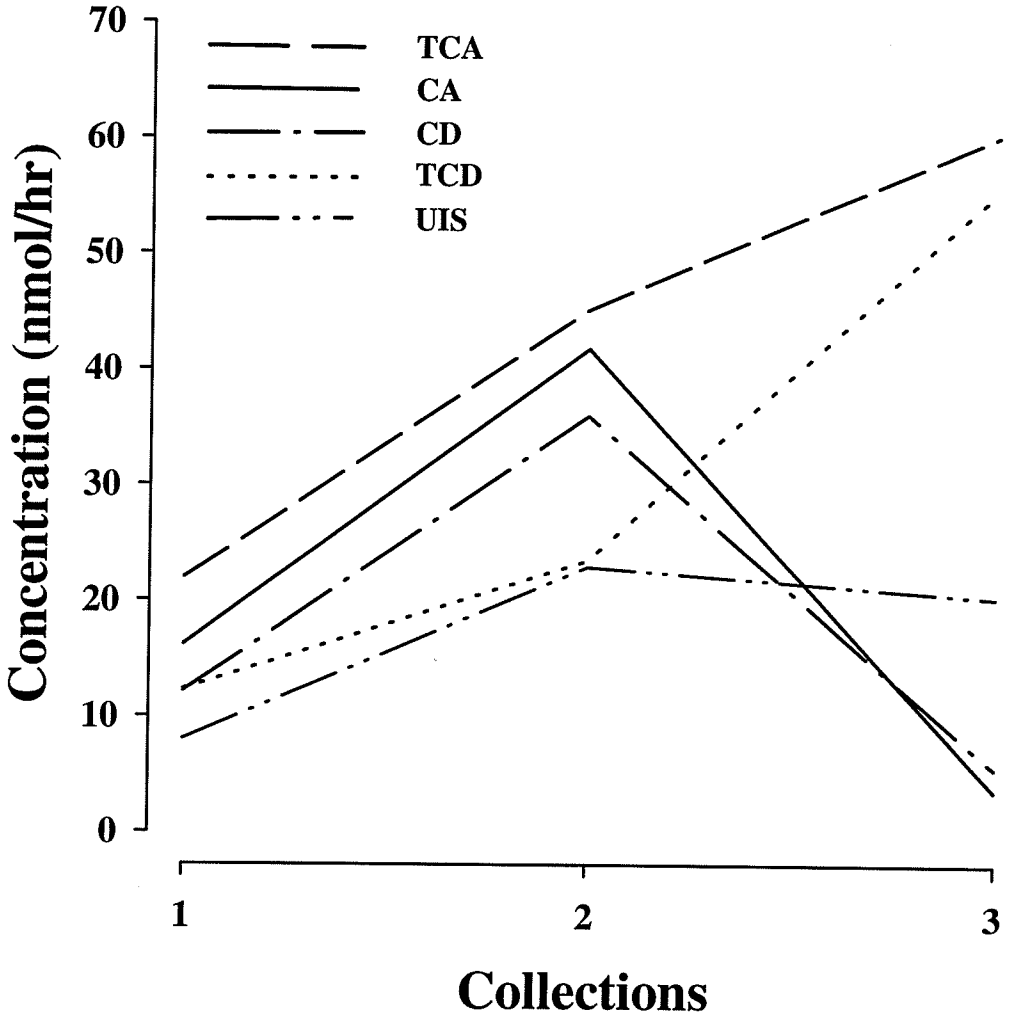


Figure 5.7 Changes in concentrations of individual bile acids in the prespawning male urine collected during three successive 12-hr periods. TCA=taurocholic acid; CA=cholic acid; CD=chenodeoxycholic acid; TCD=taurochenodeoxycholic acid; UIS=unidentified compound.

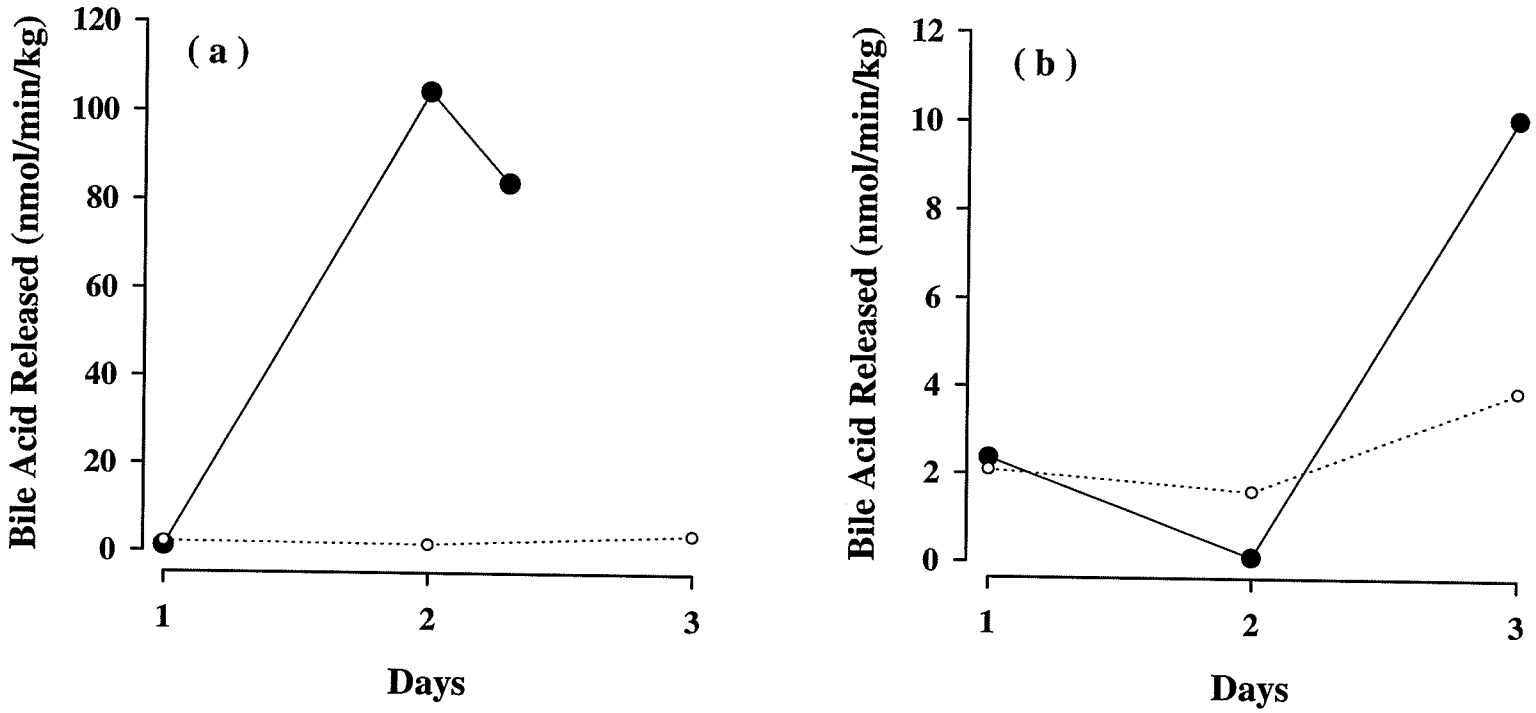


Figure 5.8 Changes in bile acid concentrations in tank water sampled on 3 consecutive days. **(a)** prespawning female ♀$_1$. **(b)** spawning female ♀$_2$. Bile acid concentrations in juvenile tank water (small open circle) are presented in both graphs for comparison. Note different Y-axis scales in (a) and (b).

concentrations in ♀$_1$ tank water was constantly higher than those in ♀$_2$ tank water. The TCA/TCD ratio in the water was approximately one half of that in the faeces (Fig. 5.5).

EOG responses to chromatographic fractions

Olfactory responses to HPLC eluted fractions of juvenile bile, faeces, urine and tank water extracts are shown in Figure 5.9. The major olfactory stimulatory components were eluted between 45-95 min for all extracts. To refine the elution time for the substances that were olfactory stimulatory, I collected ten successive eluates during this period for bile and faeces extracts and found that the elution time for major olfactory stimulatory fractions were overlapped with that of the bile acid peaks revealed in HPLC analysis (Fig. 5.10). Taurocholic acid and TCD were the two major peaks responsible for olfactory stimulation. In addition, UIS was proven a potent olfactory stimulant when the purified fraction was tested (Fig. 5.11)

Cross-adaptation was used to examine whether olfactory responses to UIS were mediated by known bile acid or amino acid receptors (Chapter 2). The adapting concentrations were at an equivalent or higher potency than the olfactory responses to UIS. Olfactory responses to UIS during adaptation to $2x10^{-8}$ M TCA, 10^{-9} M TLS, $2x10^{-8}$ M CD, $5x10^{-8}$ M PZS, 10^{-5} M CYS, and a mixture of $5x10^{-8}$ M PZS and 10^{-5} M CYS are shown in Figure 5.11. Responses to UIS remained during these adaptations. Olfactory responses to UIS during adaptation to 10^{-5} M SER was similar to those during adaptation to CYS.

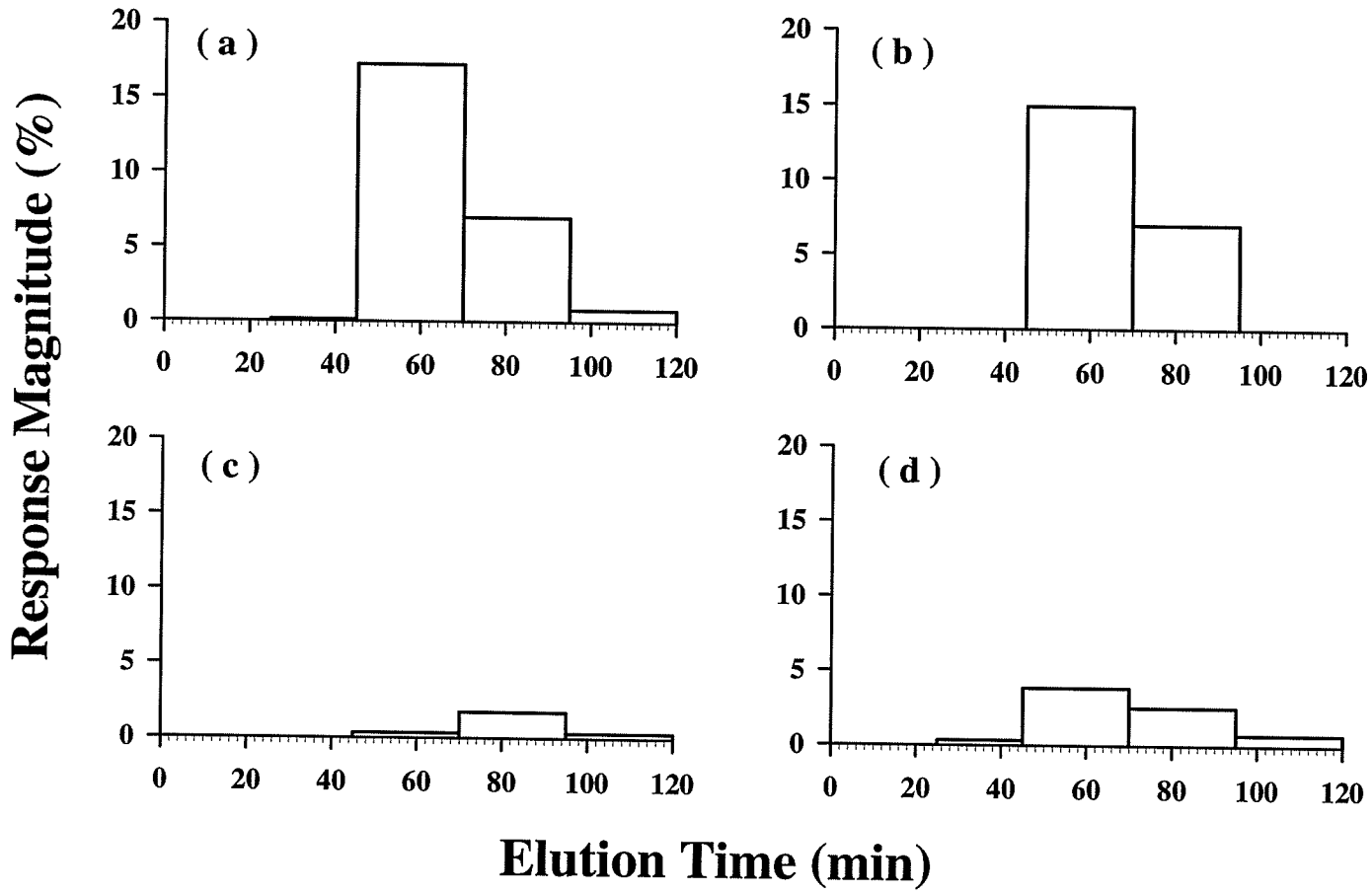


Figure 5.9 Olfactory response magnitudes to 5 successively collected HPLC fractions during 3-120 min elution time. The extracts examined were bile **(a)**, faeces **(b)**, urine **(c)** and tank water **(d)** from juveniles. The bar width indicates the eluate collection period and the height indicates the relative olfactory response magnitude to each fraction. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. The background response has been subtracted from the response magnitude. Fractions were purified as described in the text before they were applied as stimulants.

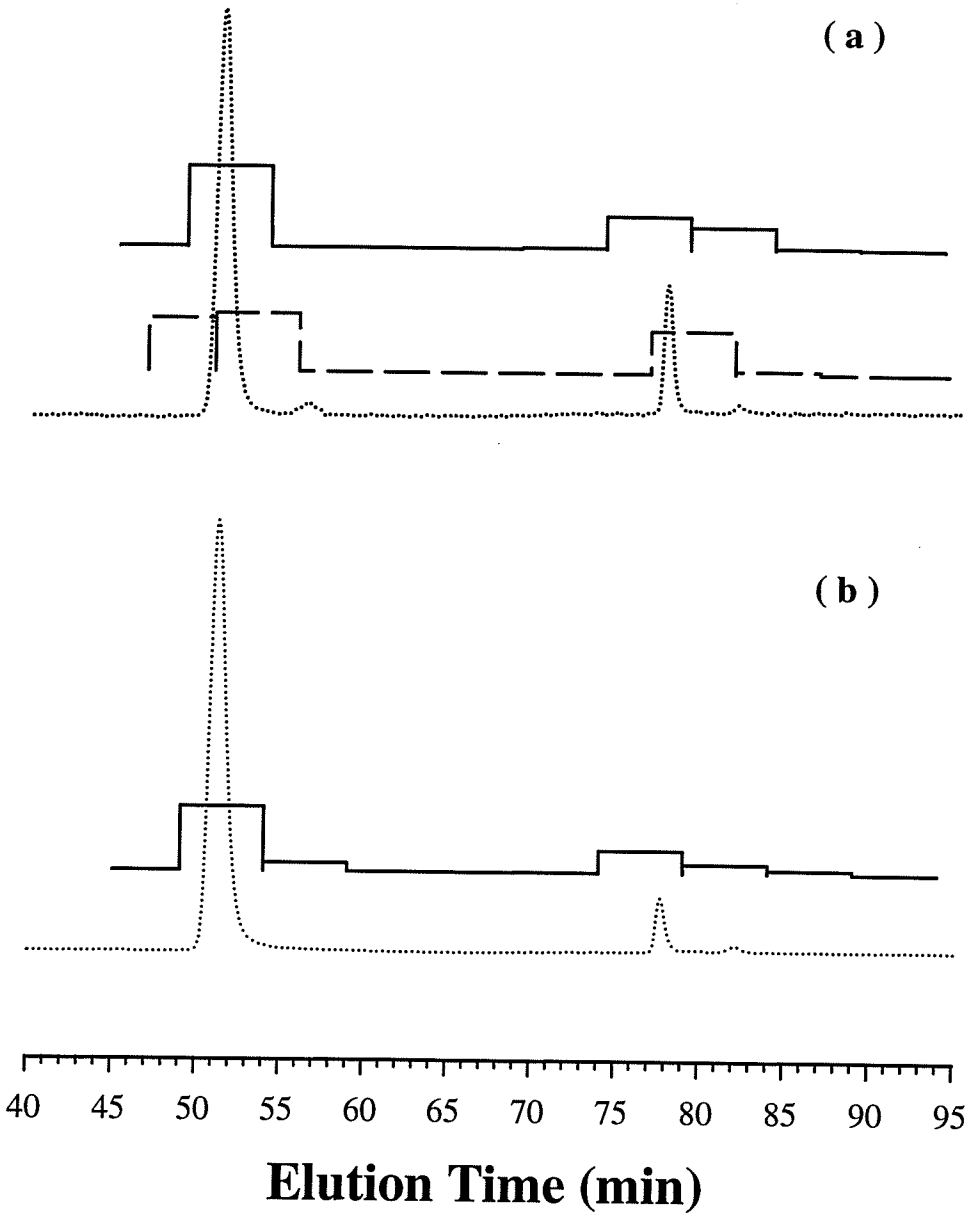


Figure 5.10 Olfactory responses to 10 successively collected HPLC fractions during 40-95 min elution time for juvenile faeces **(a)** and juvenile bile **(b)**. The bar width indicates the eluate collection period and the height indicates the relative olfactory response magnitude to each fraction. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. Differences in time of eluate collection are presented by different types of lines (solid line and broken line bars). The corresponding chromatograms are superimposed (doted line) to indicate relationships between olfactory responses to eluates and bile acid contents in the eluates.

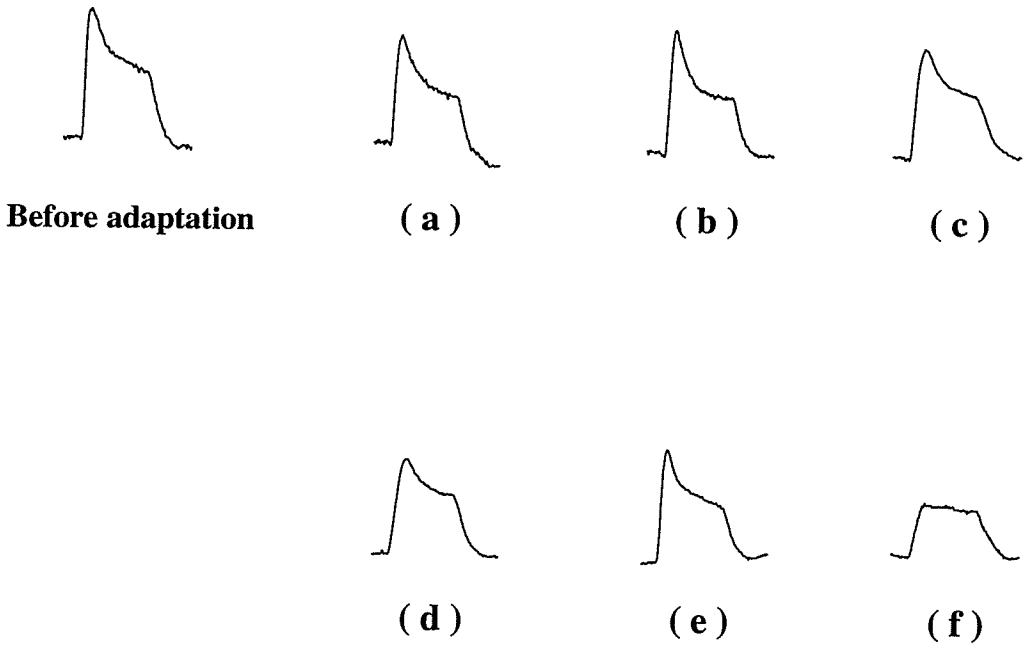


Figure 5.11 Olfactory responses to an unidentified 3α-hydroxysteroid sulphate (UIS) recorded before and during adaptation to following bile acids and the amino acid: **(a)** $2x10^{-8}$ M taurocholic acid (amidated bile acid); **(b)** 10^{-9} M taurolithocholic acid 3-sulphate (sulphated bile acid); **(c)** chenodeoxycholic acid (free bile acid); **(d)** $5x10^{-8}$ M petromyzonol sulphate; **(e)** 10^{-5} M L-cysteine; **(f)** a mixture of $5x10^{-8}$ M petromyzonol sulphate and 10^{-5} M L-cysteine.

The detection thresholds for individual extracts of the sulphated (and glucuronide) fraction (from the LH-20 chromatography) was consistent with the amount of sulphated bile acids in the extracts as determined by HPLC analyses (Table 5.4).

DISCUSSION

Analytical procedures

I have used ion-pairing HPLC combined with immobilized enzymatic post-column reactions (Okuyama et al. 1979, Baba et al. 1980; Gallaher and Franz 1990) to determine bile acids in biological samples in this study. It is a highly selective method to separate common bile acids without any chemical modification. The method has advantages in procedural simplicity, relatively high specificity, sensitivity, reliability and reproducibility. Under the defined gradient, the method can completely separate free and amidated bile acids, and is adequate for the analysis of bile acids in fish bile, faeces, urine, and tank water. When comparing relative capacity ratios for the two HPLC columns (Table 5.3), the PRP-1 column is slightly better than the econosphere C_{18} column for bile acid separation. However, the former is more expensive and separation for more polar bile acids may not be as good as the econosphere C_{18} column under the mobile phase condition used.

This HPLC measurement is specific for 3α-hydroxyl and 3α-oxo bile acids, which covers at least 90% of all bile acids and their metabolites found in vertebrates.

The results of olfactory responses to HPLC and LH-20 fractions suggest that other undetected bile acids, if present, are either in small quantities or ineffective as olfactory stimulants, which is of less interest from the chemoreception view point.

Quantitative extraction and partial separation of bile acids using C_{18} cartridges achieve high recoveries. This result agrees with the report by Whitney and Thaler (1980). The discrepancy of recoveries followed by [^{3}H]TCA and reference bile acids during sulphate ester solvolysis may result from chemical modification during the solvolysis or 1,4-dioxane effects on enzymatic reaction and fluorescence detection. It has been suggested that one of the advantages of this solvolysis method over the others is mild reaction without chemical modification (Princen et al. 1990). However, its strong acidic environment may result in methylation when these samples are redissolved in methanol. There is an indication that the detection sensitivity was affected, because the peak area of the internal standard was consistently smaller during examination of desulphated samples than that during other runs.

Bile acids compositions and biological implications

The present study has demonstrated that the proportion and composition of bile acids that lake char produced and released are consistent in individual juvenile lake char held under defined experimental conditions. Therefore, lake char are capable of maintaining bile acid homeostasis in general.

Consistent with biliary bile acid analyses in other salmonid species, lake char produce amidated CA and CD (Sasaki 1966, Haslewood 1967a, Ripatti and Sidorov

1973; Denton et al. 1974). The biliary bile acids in lake char include large amount of amidated CA and CD, as well as small amounts of ketonic bile acids, which is similar to those in rainbow trout and germfree Beagle dogs (Denton et al. 1974; Beaver 1978). In contrast to findings by Denton et al. (1974), however, glycine amidated bile acids are not detected in this study. Instead, small amounts of TLC and UIS, and approximately 7% of TOCN, which are not reported in rainbow trout, are present in the lake char bile. All biliary bile acids in lake char are either taurine amidated or sulphated, or both. This observation supports the overall evolutionary pattern proposed by Haslewood (1967b) and current concepts that non-mammals secrete exclusively taurine amidated or sulphated bile acids.

The bile acid components in faeces is the same as in bile in lake char. Common bile acid catabolites such as nonamidated DC and LC found in the mammalian are not present. A similar result has been reported for bile acid contents in rainbow trout faeces (Sacquet et al. 1979). Therefore, the degradation of bile acids in the salmonid intestine appears insignificant. Extensive bile acid metabolism occurring in the mammalian intestine is entirely caused by intestinal microorganisms, whereas digestive enzymes have no effects (Bergström et al. 1960; Gustafsson et al. 1968; Madsen et al. 1976; Huijghebaert and Hofmann 1986). Limited bacterial species and quantities in the fish intestine (Snieszko 1957; Trust and Sparrow 1974; Trust 1975; Horsely 1973, 1977) account for the lack of metabolic transformation of bile acids.

The major differences of bile acids between bile, faeces and holding water are

proportional changes of individual bile acids. The TCA/TCD ratio is systematically decreased from bile to tank water in both juveniles and adults (Fig. 5.5). Although faeces are not the only source for bile acids in the water, proportional decreases of TCA/TCD ratios in bile, faeces and tank water suggest that like the mammalian (ref. Hofmann 1994b), the fish intestine has active bile acid uptake systems to selectively transport bile acids. Similarly, these systems seem more active in the distal intestinal tract and favour more polar bile acids (i.e. TCA > TCD).

Notable differences of TCA/TCD ratio were also found between samples from juveniles and adults. The TCA/TCD ratio in the bile of juvenile lake char coincides with that of yearling rainbow trout (Denton et al. 1974). Their data also show that amidated CA/CD ratio in bile is lower for 6 month old trout than for 13 month old ones. What causes the difference of TCA/TCD ratio is presently unknown. The changes of TCA/TCD ratio do not appear to be closely related to diet, because lake char had same diet may have different TCA/TCD ratios (data not shown). The bile acid synthesis and secretion are regulated by various hormones and second messengers (e.g Thompson and Vars 1953; Bekersky and Mosbach 1973; Bennion et al. 1976; Davis and Kern 1976; Saarem and Pedersen 1987; Nathanson and Boyer 1991; Beckh et al. 1994; Chico et al. 1994; Crestani et al. 1995). For example, in mammals, high levels of thyroid and growth hormones increase the amidated CD proportion in the bile pool, whereas low levels of thyroid hormones reduce it (Eriksson 1957a, b; Heubi et al. 1983; Kosuge et al. 1987). Thyroid hormones are elevated in early gonadal development, and maintained or enhanced during the period of reproduction in fish

(ref. Cyr and Eales 1996). Growth hormone regulates puberty and gonad development. The circulating growth hormone is high during the final stage of the reproductive cycle in teleosts (ref. Le Gac et al. 1993). In fact, biliary TCA/TCD ratio in a precocious lake char is only one-fourth that in other juveniles in the same tank and is more similar to that in adults (data not shown). An increase of amidated CD level in serum of healthy pregnant women, resulting in a lower amidated CA/CD ratio than the normal, has been reported (Heikkinen et al.1981). The proportional change of amidated CA/CD in pregnant cat shows the same tendency (Rådberg et al. 1988). In hamster, a lower amidated CA/CD ratio during pregnancy is due to a decrease of amidated CA secretion (Reyes and Kern 1979).

The change of TCA/TCD ratio in bile directly affects the TCA/TCD ratio in the water (Fig. 5.5). However, this change may be of little significance with regard to bile acids as chemical signals in fish, as the detection of TCA and TCD are mediated by the same type of olfactory receptor (Chapter 2). Taurochenodeoxycholic acid appears slightly more potent than TCA. The significance of a lower TCA/TCD ratio in adult excretion should be considered if the quantitative discrimination of bile acids is critical for fish.

Because of efficient renal glomerular function, the urinary elimination of bile acids is a negligible route in mammals (Zins and Weiner 1968; Almé et al. 1977; Barnes et al. 1977; Burckhardt et al. 1987). Information about urinary bile acids in other vertebrates is rare. The present study is the first examination of urinary bile acids in fish. Major findings are that urinary excretion of bile acids in lake char is

sexually and temporally dependent. Considerable amounts of bile acids are eliminated via urine in the prespawning male. Large differences of urinary bile acid concentrations between males and females and between males at different stages in the reproductive cycle indicate a possible connection between the augmentation of urinary bile acid excretion and spawning activities of lake char. The finding that the prespawning male urine contains substantial amounts of free bile acids and UIS is of particular interest. Electrophysiological studies have demonstrated the presence of olfactory receptors specific for free bile acids, and CA and CD are among the most potent bile acids examined (Chapter 2). Although the chemical identity of UIS is currently unknown, electrophysiological cross-adaptation has demonstrated the presence of independent olfactory receptors for this compound (Fig. 5.11). Thus, these temporally released chemical cues could be acutely detected by lake char.

Prespawning male urine contains three nonamidated bile acids that are absent, or present in trace quantities, in other samples. Trace amount of UIS found in all samples suggests that lake char have the ability to produce or metabolize this compound. However, this process seems accelerated during a particular time of life such as reproduction. Present data have shown that urinary bile acids do not associate with seminal fluid production. Bile acid metabolite profiles in urine are the net result of metabolic reactions and transport processes in the liver, intestine and kidney (Almé et al. 1977). Because bile acid metabolism in the intestinal tract is negligible in lake char, the production and metabolism of bile acids in the liver or kidney are responsible for three nonamidated bile acids in the urine. Generally, synthesized bile acids are

amidated before entering the circulation. It might be possible that free bile acids are released before amidation. Furthermore, the enzyme that catalyzes deamidation is present in the mammalian liver and is active under certain circumstances (Usui and Yamasaki 1964; Borgström et al. 1987). The kidney is another important organ where vigorous metabolism takes place. Though there are no published data regarding the bile acid deamidation in the kidney, sulphation in the kidney contributes significant amounts of bile acid sulphates to the urine (Rice et al. 1968; Summerfield et al. 1976; Chen et al. 1978). Another possibility is that these nonamidated bile acids complete their synthesis in the kidney. Extrahepatic modification of bile acids, other than sulphation and glucuronidation, has been reported (ref. Elliott 1985), even though bile acids are considered to be synthesized in the liver exclusively. It is worth mentioning that bile acid synthetases include several members of the cytochrome P-450 family (P-450s) (e.g Björkhem et al. 1976; Hansson and Wikvall 1980; Boström and Wikvall 1982; Andersson and Jörnvall 1986; Andersson et al. 1989). A bile acid synthetase gene, mitochondrial cytochrome P-450 sterol 26-hydroxylase, is heavily expressed in rabbit adrenal gland, as well as in kidney to some extent (Andersson et al. 1989). In rainbow trout, the level of P-450s in the kidney increases during maturation and shows sex-related differences during late reproductive stages (Andersson 1990; Buhler et al. 1995). Cytochrome P-450 contents in male trunk kidney microsomes are about 30 times those in female. In addition, the P-450s in the rainbow trout kidney have similar immunological characteristics as the major constitutive P-450s in the liver (Williams and Buhler 1984).

Bile acids in water stimulate fish chemoreceptors. Juvenile lake char release an average of 3 nmol/min bile acids per kg of body weight into water. An early report suggested that anadromous Arctic char release 34 nmol/kg of 3α-hydroxysteroids per hour into the water (Døving et al. 1980). If their detection methods were specific for bile acids, the amount of bile acids that anadromous Arctic char excreted is only one fifth of those released by juvenile lake char. Analysis of the water that held individual adults shows that lake char release bile acids into water in an intermittent fashion. The temporal changes of bile acid concentrations are also observed, but to a lesser extent, in tank water that held juveniles. This temporal change prohibits quantitative comparison of bile acids excretion between individuals or species until an understanding of the release pattern is achieved. This temporal release, however, explains why a lower concentration of bile acids was detected in $♂_2$ tank water than in $♂_1$ tank water while urinary bile acid excretion is higher in ♂2, assuming that the release of bile acids via the intestine tract is the same in both fish. A high proportion of UIS in $♂_2$ tank water reflects the urinary bile acid profile of this fish. Thus the water where prespawning males live has characteristic odours. This study also indicates that lake char may release more than 340 nmol of bile acid per minute (e. g. bile acid concentrations in $♀_1$ holding water). This amount should be sufficient to be detected by conspecifics, because the physiological thresholds for TCA and TCD are around 0.5 nmol/l (Chapter 2).

Where do the large amounts of bile acids in the female holding water come from? Female urine contains negligible amounts of bile acids. It is unknown whether

fasting fish still release considerable amounts of bile acids via the intestinal fluid and in what amount. In humans, about half the synthesized bile acids bypass the gall bladder and enter the intestine during overnight fasting (Northfield and Hofmann 1975; van Berge Henegouwen and Hofmann 1978; Mok et al. 1980). The gall bladder contracts even in the interdigestive state (Itoh and Takahashi 1981). In their study of enterohepatic circulation of thyroxines in brook trout, Eales and Sinclair (1974) showed that losses of [^{125}I]T_4 in the gall bladder were one order of magnitude faster in fed than in starved fish. Presumably, differences of bile acid discharge to the intestine between fed and starved fish would be close to this figure. In the present study, the bile acid profile in the faeces was from 3-day fasted fish. If starved fish release bile acids through the intestinal tract, their profile should be similar to the bile acid composition in faeces. Gills may provide another route for bile acid release. Gonadal steroids, especially conjugated gonadal steroids, and thyroid hormones released or absorbed through gills have been reported (Maren et al. 1968; Eales 1974; Omeljaniuk and Eales 1985; Budworth and Senger 1993; Scott and Vermeirssen 1994). Using an apparatus to separate and simultaneously collect the excretion emitted from gills, faeces and urine (Post et al. 1965; Maren 1968) may provide some answers.

In addition to the fact that the prespawning male excretes nonamidated bile acids, all lake char examined, including juveniles, release TCA and TCD into the water. It is expected that other salmonid species also release considerable amount of TCA and/or TCD into the water, for their biliary bile acids are mainly TCA and/or TCD (Sasaki 1966, Haslewood 1967a, Ripatti and Sidorov 1973; Denton et al. 1974).

This reality does not necessarily conflict with the hypothesis that bile acids may function as chemical cues in reproduction (present data) and migration (Døving et al. 1980; Li et al. 1995), although the role of bile acids in mediating fish behaviour deserves further studies. In lake char, considerable amount of nonamidated bile acids are released during the spawning season. Lake char can discriminate these compounds from TCA and TCD. Although homing or migration is an inherent characteristics for many salmonids, the original strategy for spawning migration is probably to seek a suitable environment for offspring, including rock debris or gravel, well-oxygenated and flowing water, and food resource for alevins. Optimal conditions for spawning and for offspring growth are similar among many salmonids (Drummond Sedgwick 1986). Homing or migration cues may be common to all salmonids.

Some steroid glucuronides may be pheromones for black goby (Colombo et al. 1980), zebrafish (van den Hurk and Lambert 1983; van den Hurk et al. 1987), African catfish (Resink et al. 1989a, b; van den Hurk and Resink 1992) and the herring *Clupea harengus pallasi* (Sherwood et al. 1991). Ovarian and/or seminal fluids have been implicated as the source of chemical cues in several fish species (Rubec and Thomas 1979; ref. Liley 1982; van den Hurk and Lambert 1983; Kobayashi et al. 1986; van den Hurk 1987; Resink et al. 1989a, b; van den Hurk and Resink 1992). In the present study, glucuronide conjugated bile acids were not detected in all samples examined. Also, gonadal fluids do not contain bile acids. If pheromonal substances for lake char are glucuronide conjugated compounds or of gonadal origin, these compounds should not be bile acid-like substances. The role of bile acids involved in

the function of other pheromonal substances is of interest for future study.

SUMMARY

This is a systematic study of production, metabolite profiles and excretory routes of bile acids in lake char. The following results generally agree with findings in mammals: 1) CA and CD are the principal bile acids lake char synthesized, 2) faeces are the major pathway for bile acid elimination under normal conditions, 3) urinary excretion is of lesser importance, and 4) glucuronidation is an insignificant conjugation route for bile acids. However, salmonids have a much simpler bile acid metabolite profile than mammals, largely due to a paltry microflora in the fish intestinal tract. Fish may thus serve as a good experimental model to study bile acid metabolism without the interference of microflora under natural physiological conditions.

This study has demonstrated that olfactory stimulants in extracts from juvenile bile, faeces, urine and tank water are directly associated with the bile acid components that were determined by HPLC analysis. The data have indicated the importance of bile acids in natural excretions that may serve as chemical signals. One microlitre of bile or 10 mg faeces in a thousand litres of water could be detected by fish olfaction. Prespawning male urine is very "odorous". One minute of urinary excretion in the prespawning male is equivalent to 1 μl bile or 10 mg faeces from juveniles. The majority of bile acids found in biological samples is among the most stimulatory for

olfaction and can be distinguished from one another by specific olfactory receptors (Chapter 2). Thus, lake char can detect and discriminate various bile acids released by their conspecifics. The composition and concentration changes of bile acid excretion in prespawning fish are of particular interest if bile acids function as chemical signals during spawning. My study has not only presented new and substantial evidence on the production and excretion of bile acids in lake char, but also shed light on directions for further studies on bile acids as chemical signals in fish.

CHAPTER 6

Summary and Conclusions

This research is aimed to examine the pheromonal role of bile acids in lake char. Pheromones are "substances which are excreted to the outside by an individual and received by a second individual of same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process" (Karlson and Lüscher 1959). Based on this definition, water-borne chemicals may be considered as fish pheromones (or chemical signals) if these compounds are released by fish, detected by their conspecific chemosensory systems and eventually induce physiological and/or behavioural responses in fish. The data presented in this thesis support the idea that bile acids are this type of biological substance that meets the three basic criteria as chemical signals for lake char (Chapter 2-5).

Lake char possess high olfactory sensitivity and specificity to bile acids. Olfactory responses to bile acids are mediated by receptors and their underlying transduction mechanisms that differ from those for other known olfactory stimuli, amino acids, PGs and gonadal steroids (Chapter 2). Among the 38 bile acids examined, 9 of which are stimulatory at sub-nanomolar concentrations. Olfactory responses to the 9 most stimulatory bile acids are mediated by free, amidated and sulphated bile acid receptors. These receptors recognize whether a bile acid is modified by conjugations, whether the conjugation is amidation or sulphation, and where the position of sulphate ester conjugation is. Bile acids with 7α- and/or 12α-

hydroxyls on sterol rings are important to interact effectively with free or amidated bile acid receptors. Bile acids with 3α-hydroxyl sulphate ester conjugation and taurine amidation are optimal substrates for sulphated bile acid receptors. Lake char excrete TCA, TCD, CA, CD and TODC sulphate (Chapter 5). These bile acids have stereo structures that are among the most effective substrates for free, amidated or sulphated bile acid receptors. Lake char, especially prespawning males, also excrete an unidentified 3α-hydroxysteroid sulphate -- UIS. The presence of another type of olfactory receptor specific for UIS is also suggested.

Characterization of bile acid receptors, using electrophysiological cross-adaptation method and receptor autoradiographic binding, has demonstrated that bile acids interact with the receptors in a saturable, reversible and stereospecific pattern (Chapter 2, 3). Receptor autoradiography studies have indicated the presence of specific bile acid receptors on olfactory sensory neurons (Chapter 3). The majority of bile acid receptors is located on the cilia of sensory neurons. Bile acid receptors in the olfactory system are different from those in the vertebrate digestive system. Thus, it is conceivable that fish have the capability to detect and discriminate low concentration bile acids in the surrounding water. This capability is mediated by highly specific receptors and transduction mechanisms in the lake char olfactory system. The perception of bile acids induces lake char behaviour responses (Chapter 4).

HPLC analyses using immobilized 3α-HSD enzymatic post-column reactions have demonstrated the composition and concentrations of bile acids in bile, faeces,

urine and tank water from juvenile and adult lake char (Chapter 5). These quantitative data indicate that fish may detect and discriminate biological excretions in the water by responding to bile acid components in the excretions. Although lake char are able to maintain bile acid homeostasis, composition and concentration changes of urinary bile acid excretion between genders and same genders at different stage of reproductive cycle were observed. Nonamidated bile acids (CA, CD and UIS) released by prespawning males can be acutely discriminated from other types of bile acids through various bile acid receptors in the olfactory epithelium. These bile acids may act as status signals for readiness of spawning.

This hypothesis is supported by several observations. Field studies have shown that male lake char aggregated at the spawning area a week or more before females arrived (Miller and Kennedy 1948; Martin 1957; McCrimmon 1958; DeRoche 1969). The presence of ripe fish appears important to attract lake char for selecting a spawning ground (Martin 1960). The reproductive status of the prespawning male ($♂_2$) in my experiments (Chapter 5) coincides with this timing. Another compelling correlation is that the olfactory sensitivity to free bile acids is consistently higher in lake char than in rainbow trout and zebrafish (Michel and Lubomudrov 1995; Zhang and Hara, unpublished). Detection of nonamidated bile acids that may act as chemical signals during the spawning season appears very important for lake char.

Using a combination of bile acids that were identified in Chapter 5 as stimulants to examine the reproductive physiological and behavioural responses of prespawning female lake char will be the next approach for further study. Lake char

release bile acids into the water in a pulsatile fashion. We have to be careful as to whether the rhythm of release itself is taken into account as communication language. The use of a mixture of stimuli is challenging because the optimal proportion of individual components could be critical for fish to react. Considering the fact that these active compounds may constitute only a part of olfactory stimuli in a sea of chemicals in nature, we are facing further challenges to determine how much bile acid signals contribute to the entire olfactory image for fish at different life stages and whether other pieces of information are required for fish to integrate and interpret bile acid signals.

Electrophysiological approaches to examine chemostimulants and their receptors, and biochemical methods to identify the chemical nature of fish excretions are powerful for studying putative pheromones in fish, as have been proven here. However, we know little of how pheromonal information is processed after being transmitted to the central nervous system, and where is the central station at which various information from different chemosensory systems is integrated. When we can answer these questions, we will have a better understanding on how chemical signals induce physiological and behavioural responses in fish. I see my present studies as a firm step towards these investigations. I am confident in future research of chemoreception as more new techniques become available.

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