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CHARACTERIZATION OF A SPAWNING PHEROMONE OF PACIFIC HERRING

by

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

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this thesis as conforming to the required standard

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ABSTRACT

Pacific herring, *Clupea harengus pallasi*, possesses a pheromone in the milt and testes that triggers spawning behaviour in reproductively mature individuals of both sexes, and plays a role in synchronizing the school spawning that is distinctive of this species. The pheromone was found to be effective as a transient olfactory stimulus in eliciting a behavioural response that varied in the degree of expression and time course. Stimulus strength was found to influence the time course of the response, whereas differences in maturity, evident through examination of plasma levels of steroids, were correlated with a propensity to respond to the pheromone. Input from factors other than the spawning pheromone appear to be needed to elicit prolonged spawning; some of these factors also act through olfaction. Immediate effects of stress were not found to influence the response to the spawning pheromone.

Plasma levels of reproductive steroids of herring during the spawning season were measured with radioimmunoassays. Peak levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were found to coincide with final maturation in females and the initiation of milt production in males, suggesting that this steroid is the maturation-inducing steroid of this species. Other features found to be distinctive of the reproductive physiology of the herring included low plasma levels of the unconjugated maturation-inducing steroid, high levels of 17α-progesterone (17-P) and 3α,17α-dihydroxy-5β-pregnan-20-one (3α,17-P-5β), and high levels of glucuronated steroids. Structural investigation of the pheromone with liquid chromatography/ mass spectrometry showed that it consists of at least two components which do not elicit a behavioural response individually. One of these compounds is sulphated 17,20β-P.

The structure of proteinaceous hormones involved in controlling reproduction of the herring was also investigated. It was shown that this species possesses three forms of gonadotropin-releasing hormone (GnRH) in the brain, one with a structure that has not been reported before. These results indicate that the presence of three GnRH forms is a primitive, rather than derived, condition in the teleosts. The structure of the β-subunit of gonadotropin II (GtH II-β) of herring was also deduced by isolation of a cDNA for this molecule. The structure of the herring GtH II-β was found to be quite different from other teleost molecules of this kind, and a phylogenetic analysis of known GtH II-β structures suggests that the β-subunit of both mammalian gonadotropins may be most closely related to the β-subunit of teleost GtH-I.

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LIST OF ABBREVIATIONS

Chemical names:

11-DOC: 11-deoxycortisol, or 21-hydroxy-4-pregnen-3,20-dione

11-KT: 11-ketotestosterone

3α,17-P-5β: 3α,17α-dihydroxy-5β-pregnan-20-one

17-P: 17α-hydroxyprogesterone or 17α-hydroxy-4-pregnen-3,20-dione

17,20α-P: 17α, 20α-dihydroxy-4-pregnen-3-one

17,20β-P: 17α, 20ß -dihydroxyprogesterone or 17α, 20ß-dihydroxy-4-pregnen-3-one

17α,20β,21-P: 17α,20β,21-trihydroxyprogesterone or 17α,20β,21-hydroxy-4-pregnen-3-one

A: androstenedione

ACN: acetonitrile

BSA: bovine serum albumin

DCM: dichloromethane

EGMEE: ethylene glycol monoethyl ether

E: estradiol

FSH: follicle stimulating hormone

GnRH: gonadotropin releasing hormone

GtH: gonadotropin

LH: luteinizing hormone

MIS: maturation inducing steroid

RNA: ribonucleic acid

T: testosterone

TEAF: triethylammonium formate

TEAP: triethylammonium phosphate

TFA: triflouroacidic acid

Other terms:

+ve: responsive to pheromone (behaviour); positive ionization (MS)

-ve: not responsive to pheromone (behaviour); negative ionization (MS)

cDNA: complementary DNA

CID: collisionally-induced dissociation;

ESI-MS: mass spectrometry with an electrospray ionization source;

ESI: electrospray ionization;

FIA: flow-injection analysis;

g.e.: gram equivalent;

GC: gas chromatography;

GSI: gonadosomatic index

HPLC: high pressure liquid chromatography;

LC/ESI-MS: linked liquid chromatography-mass spectrometry with an electrospray interface;

LC: liquid chromatography;

LH-20: LH-20 Sephadex gel;

MALDI/MS, matrix assisted laser desporption mass spectrometry

MRM: multiple reaction monitoring;

MS/MS: tandem mass spectrometry.

MS: mass spectrometry;

NJ: neighbour joining

PCR: polymerase chain reaction

UTR: untranslated region

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CHAPTER 1: OVERVIEW OF REPRODUCTIVE PHEROMONES AND THE ROLE OF HERRING RESEARCH

Pheromones are compounds that are secreted to the exterior by one organism to induce a specific reaction in a conspecific (Karlson and Lüscher, 1959). These participate in a wide variety of interactions amongst individuals of many species, ranging from protozoans and algae to vertebrates, and have been described as the "first signals put to service in the evolution of life" (Wilson, 1975),. Pheromonal effects have been reported for reproduction, developmental synchronisation, social structuring, alarm signalling, trail marking, territory marking, dispersion, aggregation, aggression, and schooling. These effects include both the modification of physiological processes and the triggering of behaviours, classified as "priming" and "releasing" pheromones, respectively (Wilson, 1963).

Structural identification of pheromones is important for elucidating the mechanisms of pheromonal communication, but their low natural concentration after secretion has made structural determination difficult. Nevertheless, starting with the identification of bombykol in the silk moth (Butenandt et al., 1959), pheromones of a variety of animals have now been identified, many of them pheromones with reproductive function ("reproductive pheromones"; Table 1). Releasing pheromones of insects have been particularly amenable to study, largely due to the availability of practical bioassays (Blum, 1985; Struble and Arn, 1984, Vogt, 1987). The picture that has emerged for this group of animals is that their pheromones are generally a blend of compounds, in which both the

Table 1: Chemical nature of representative reproductive pheromones

Chemical class	Compound(s)	Precursor	Source	Occurrence	Sex[1]	Function[1]	Ref[2]	Comments[1]
fatty acids								
	prostaglandins (PGs)	PG hormones?	gonad?	teleosts	m/f	induce spawning	14	
	short chain FA		vaginal discharge	primates	f	excite m		blend
long chain HC								
	alcohols aldehydes acetates	fatty acids	epidermal glands	Lepidoptera	f	attract m	2	blends; evolutionary trends
	ketones & acetates	-	urine	mouse	f	inhibit f maturation	5	
	methyl ketones	skin lipids	integument	garter snake	f	elicit m mating	10	blend
	muscalure	fatty acids & acetate	cuticular	house fly	f	attraction	3	
terpenes								
	mono-terp	acetate & external sources	epidermal glands	insects	m/f	attraction	3	
	farnescene	-	epidermal glands	mouse	m	dominance	11	
	squalene		skin	snakes	f	m copulation	9	blend
	steroids & conjugates	maturation hormone	gonad?	teleosts	f	induce final maturation	14	
	sesquiterpenediol	sirenin	-	fungus	f	attract sperm	7	m pheromone similar
	pyrazine	-	urine	mouse	f	inhibit f maturation	5	blend
	thiazole	-	urine	mouse	m	Whitten effect	11	blend w/ farnescenes
	brevicom	-	urine	mouse	m	Whitten effect	11	blend w/ farnescenes
	5α-androstenone & 3α-androstenol	pregnenolone	urine & musk	pig	m	f mating behavior	12	possibly part of blend
aromatic								
	phenylethyl alcohol	phenylalanine	epidermal gland	insect	m	induce f copulation	3	
	dichlorophenol	tyrosine?	epidermal	ticks	f	m attraction	3	
	vanillic acid			nematode	f	m attraction	4	single compound

Table 1: Chemical nature of representative reproductive pheromones (Cont'd)

Chemical class	Compound(s)	Precursor	Source	Occurrence	Sex[1]	Function[1]	Ref[2]	Comments[1]
Proteins								
	aphrodisn	-	vaginal?	hamster	f	m copulation	13	related to carrier protein
	α-mating factor	-	-	yeast	-	induces mating	6	contains GnRH-like segment
Nucleotides								
	cAMP			slime mold		attraction	1	
Others								
	cyclopropanes etc.	fatty acid fragment	-	brown algae	f	sperm attraction	8	taxonomic trends evident
	dimethyl sulfate	-	-	hamster	f	m attraction	13	
	alkaloids	plant alkaloids	epidermal hairs	Lepidoptera	m	f copulation	3	

[1] m=male, f=female

[2] References: 1. Bonner, 1963; 2. Blum, 1985; 3. Blum, 1987; 4. Jaffe et al., 1989; 5. Jemilio et al., 1986; 6. LeRoith & Roth, 1984; 7. Machils et al., 1968; 8. Maier & Muller, 1986; 9. Mason et al., 1989; 10. Mason et al., 1990; 11. Novotny et al., 1990; 12. Reed et al., 1974; 13. Singer et al., 1987; 14. Sorensen & Stacey, 1990.

chemical nature and relative concentration of the various components contributes to the potency and species-specificity.

Evolutionary relationships amongst the chemical structures of pheromones is evident in closely related species, but not among those of more distantly related insects or other organisms (Table 1). Sensory mechanisms and transduction pathways of the pheromonal signals, on the other hand, appear similar, though homology has not been clearly demonstrated. As represented by moths and butterflies, the response mechanism to pheromones consists of 1) finely tuned receptor cells in sensillary hairs of antennules, and 2) dedicated "labelled-line" primary axons leading to specialized macroglomeruli in antennal lobes (Mustaparta, 1984). Details of the subsequent neural mechanisms responsible for distinctive behavioural responses to the pheromonal input are still poorly understood, but also appear to have pheromone-specific pathways in some cases (Hansson, 1995).

Vertebrate pheromones have yielded less readily to structural identification, possibly because these pheromones tend to elicit more subtle influences on physiology or behaviour than the insect releasing pheromones, often in concert with other cues or associative learning (Singer et al., 1987; Wilson, 1970). Hence, effective bioassays are often more difficult to establish. However, reproductive pheromones of a variety of vertebrates are now known (Table 1). These include priming pheromones of mice and rats (see Novotny et al., 1990), and releasing pheromones of snakes (Mason et al., 1989) and hamsters (Singer et al., 1987). Chemical signalling for a variety of functions is likely to be important to many other vertebrates as well (Wilson, 1975), but a lack of information on the chemical identity of the pheromones has slowed research on specific reactions. Present evidence suggests that, as in

insects, pheromones of vertebrates are diverse in structure (Table 1), while transduction mechanisms may be homologous to one another. The "vomeronasal" organ is an olfactory structure that is found in most tetrapods, and is believed to be primarily involved in pheromone detection (Bertmar, 1981; Eisthen, 1992; Dorries et al., 1997).

Fish are representatives of a basal vertebrate lineage predating the evolution of vomeronasal organs. However, pheromonal communication in these vertebrates is also well developed (e.g. Colombo, et al., 1982; Sorensen, 1992; Table 2) and morphological specialization for olfactory detection of pheromones appears to be present (Dulka, 1993). The reproductive pheromones of fish are of particular interest in that many appear to be related to reproductive hormones that are distinctive of the stage of reproduction when the pheromonal communication is most appropriate (Stacey and Sorensen, 1991). Thus, these pheromones may represent an early stage in the evolution of pheromonal communication as described by Wynne-Edwards (1962), who suggested that "all functional odors have evolved from metabolites originally secreted for another function". Studies of "primitive" pheromonal systems such as those found in fish reproduction may provide an understanding of pheromonal communication in vertebrates.

Spawning of the Pacific herring occurs in large schools without noticeable pairing between sexes or direct behavioural interaction between individuals (Schaeffer, 1937), which is thought to be characteristic of a primitive reproductive strategy in fish (Keenleyside, 1979). A spawning pheromone involved in synchronization of spawning of herring (Stacey and Hourston, 1982; Sherwood et al., 1991; Carolsfeld et al., 1992) is unusual in that it is produced by males but rapidly elicits spawning in individuals of both sexes. Most other reproductive

pheromones of fish that have been studied elicit a less clear physiological or behavioural response and act primarily on only the opposite sex (Table 2). The herring pheromone may thus provide an informative contrast to pheromones of other fish, and help answer some of the principal questions now challenging this field of research as described below.

1) Are fish pheromones specialized signals for communication?

Communication has been defined by Wilson (1975) and others as a bilateral process in which both the signaller and the recipient participate in a specialized manner specific to the act of communicating. Controlled release of reproductive pheromones of fish specifically for communication, however, has rarely been demonstrated. Rather, a physiological or behavioural response to the smell of hormones and hormone metabolites excreted during particular reproductive states appears to have evolved in the recipient without specific specialization of the signal by the emittor (Sorensen and Scott, 1994). This level of interaction has been termed "spying", and may be an evolutionary precursor of true chemical communication, in which specialization of both the release and the reception of a signal exists (Stacey and Sorensen, 1991).

We do not yet know if the spawning pheromone of herring consists of compounds produced specifically for communication. The male African catfish, blenny and black goby attract females with androgenic compounds produced in specialized structures (Resink, 1988; Laumen et al., 1974; Colombo et al., 1980). These may be rare teleost examples of true communication with reproductive pheromones. No specialized pheromone-producing structure has been found in Pacific herring.

Table 2. Representative pheromones of fish

Pheromone Type	Species	Source Tissue	Sex[1]	Maturity	Response	Sex[1]	Structure[2]	Ref[3]
Releasing:								
Alarm								
Ostariophysii	minnows & others	skin	m & f	all	anti-predator	m & f		22, 32
Kin recognition								
Cichlasoma nigrofasciatum	cichlid		m & f	brooding	recognition of young	m & f		17
Ictalurids	catfish	skin mucous	m & f	all	recognition	m & f	neuropeptides?	5
Oncorhynchus kisutch	coho salmon	skin mucous	m & f	all	homing	m & f		7, 13
Petromyzon marinus	sea lamprey	bile	m & f	larvae	homing	m & f	bile acids	16
Phoxinus phoxinus	minnow	skin mucous	m & f	all	attraction for schooling	m & f		6, 33
Plotosus lineatus	marine catfish		m & f	all	attraction for schooling	m & f		9
Reproductive								
Acheilognanthus lanceolatus	slender bitterling	ovarian fluid	f	ovulated	attraction	m		11
Bathygobius soporator	frillfin goby	ovarian fluid	f	ovulated	courting	m		29
Belontiids			f	ovulated	attraction	m		15
Belontiids			m		attraction	f		15
Blennius pavo	blenny		m		attraction	f		14
Brachydanio rerio	zebrafish	ovary	f	ovulated	attraction & courtship	m	steroid glucuronides	30
Brachydanio rerio	zebrafish		m		attraction	f	steroid glucuronides	1, 31
Carassius auratus	goldfish	ovarian fluid	f	ovulated	attraction	m	ether soluble	20
Carassius auratus	Goldfish	urine	f	ripe	courting	m	15keto-$PGF_{2\alpha}$	24
Clarias gariepinus	African catfish	seminal vesicles	m		attraction	f	3α,17-P-5β?	31
Clupea harengus pallasi	Pacific herring	milt	m	spermiated	spawning	m & f	steroid conjugates	21, 26
Gobius jozo	black goby	mesorchial	m		attraction & oviposition	f	etiocholanolone gluc	4
Haplochromis burtoni	cichlid		f	gravid	courting	m		7
Hypomesus olidus	pondsmelt	ovarian fluid	f		courtship	m		19
Hypomesus olidus	pondsmelt	testes	m			f		19
Hypomesus olidus	pondsmelt	ovarian fluid	f		courtship	m		19
Misgurnus anguillicaudatus	loach	ovarian fluid	f	ovulated	attraction	m		12
Oncorhynchus masou	yamame salmon	ovarian fluid	f	ovulated	attraction	m		12
Oncorhynchus rhodurus	amago salmon	ovarian fluid	f	ovulated	attraction	m		12
Plecoglossus altivelis	ayu	ovarian fluid	f	ovulated	attraction	m	water-ether soluble	10
Poecilia chica	guppy		f					
Poecilia reticulata	guppy		f		attraction & courting	m		28
Rhodeus ocellatus	rose bitterling	ovarian fluid	f	ovulated	attraction	m		11
Salmo gairdneri	Rainbow trout	ovarian fluid	f	ovulated	attraction	m	water-ether soluble	11
Salmo gairdneri	Rainbow trout		m		attraction	f		18
Sarotherodon mossambicus	tilapia		f	ovulated	courting	m		28

Table 2. Representative pheromones of fish (Cont'd)

Pheromone Type	Species	Source Tissue	Sex[1]	Maturity	Response	Sex[1]	Structure[2]	Ref[3]
Priming:								
Reproductive								
Brachydanio rerio	zebrafish		m		ovarian growth & ovulati	f		2
Carassius auratus	Goldfish	ovary/urine	f	mature	spermiation	m	17,20β-P; 17,20β-P su	25, 27
Pterophyllum scalare	cichlid		m		ovarian growth & ovulati	f		3
Growth								
various					growth	m & f	lipid-soluble	5, 23

[1] f = female; m = male

[2] 15 keto-$PGF_{2\alpha}$ = 15-keto-prostaglandin $F_{2\alpha}$; 3α17-P-5β = 3α, 17α-dihydroxy-5-pregan-20-one; 17,20β-P = 17α, 20β-dihydroxy-4-pregnen-3-one

[3] References: 1. Bloom and Perlmutter, 1977; 2. Chen & Martinich, 1975; 3.Chien, 1973; 4.Colombo et al., 1980; 5. Colombo et al., 1982 (review); 6. Crapon de Crapona, 198 7. Doving et al., 1974; 8. Goz, 1941; 9. Hayashi et al., 1994; 10. Honda, 1979; 11. Honda, 1980a; 12. Honda, 1980b; 13. Johnson & Halser, 1980; 14. Laumen et al., 1974 15. Lee & Ingersoll, 1979; 16. Li et al., 1995; 17. Myrberg, 1975; 18. Newcombe & Hartmann, 1973; 19. Okada et al., 1978; 20. Partridge et al., 1976; 21. Sherwood et al. 22. Smith, 1992 (review); 23. Solomon, 1977; 24. Sorensen et al., 1996 (review); 25. Sorensen, 1996 (review); 26. Stacey & Hourston, 1982; 27. Stacey & Sorensen, 1986 28. Stacey et al., 1986; 29. Tavolga, 1956; 30. Van den Hurk & Lambert, 1983; 31. Van den Hurk & Resink, 1992 (review); 32. von Frisch, 1941; 33. Wrede, 1932.

Structural elucidation of the spawning pheromone should help resolve the type of chemical interaction that is used by herring.

2) How are teleost pheromones species specific?

The majority of reproductive pheromones identified in fish so far are steroids. However, many of the steroid hormones associated with fish reproduction are common to a variety of fish species, many of which may be sympatric. This raises the problem of whether an unspecialized pheromone elicits heterospecific responses. Most of the pheromones investigated are associated with the relatively short period of final maturation and/or spawning, so potential heterospecific responses may be limited to fish that are in an appropriate physiological state and in close proximity. Resink (1988), however, also describes pheromones of African catfish that are active during the more prolonged period of vitellogenesis. So far, a definitive mechanism has not been discovered that explains why individuals of other species do not also respond to a pheromone-emitting fish, but several hypotheses have been developed. Van Weerd and Richter (1991) and Stacey and Sorensen (1991) suggest that the steroids of interest are metabolised by sufficiently different pathways that a species-specific signal is readily formed from individual steroids, mixtures of steroids, or mixtures of steroids with non-hormonal components. In fact, while most pheromones studied so far contain compounds that have pheromonal activity on their own, the natural pheromone probably consists of a mixture of compounds (Colombo et al., 1982; Sorensen, 1996; Van Weerd and Richter, 1991). A species-specific pheromone signal of this kind could exist without specialization of the signal for communication, particularly if selective olfactory sensitivity

evolves in the responding fish (Sorensen et al., 1990). Sorensen and Stacey (1990) also suggest that pheromone-containing urine may be secreted in a species-specific pulsatile fashion. This regulated mode of emission would provide a mechanism for true communication.

Herring milt containing pheromone is present in substantial quantities in the water of a spawning school. The chemical identity of the pheromone components may indicate how the pheromone is species-specific, if the structures are unique. In addition, an analysis of the factors regulating responsiveness to the pheromone in herring should provide important clues as to how the pheromone contributes to "school" spawning of the species.

3) How does reproductive physiology influence pheromonal communication?

Both "priming" pheromones that trigger final steps of maturation of the reproductive system and "releasing" pheromones that attract the opposite sex or enhance spawning behaviour have been described for teleosts (Van Weerd and Richter, 1991). The "hormonal pheromone" hypothesis for fish (Stacey and Sorensen, 1991) suggests that pheromones signal the reproductive state of the emittor by their content of hormone-related substances. Known priming pheromones of females are related to the steroid involved in the final maturation of oocytes, as predicted by the hypothesis. However, the hormonal control of reproduction of male fish is not as well understood as that of female fish, so the relationship of these pheromones to reproductive physiology is less clear. Priming pheromones of the male zebrafish are produced in the testes (Van Den Hurk et al., 1987) and a pheromone of the male Baikal

sculpin is related to testosterone, a steroid important in male maturation (Stacey and Sorensen, 1991).

Spawning of females is regulated by prostaglandins in the goldfish, so prostaglandins can be expected to be female releasing pheromone for eliciting spawning behaviour of male fish. However, releaser pheromones that have been found are more diverse: the female goldfish and arctic char pheromones (Sorensen et al., 1988; Svensen, pers. com.) are prostaglandins, whereas the female zebrafish pheromone contains estradiol and testosterone metabolites (Van den Hurk and Lambert, 1983) and the pheromone of female guppies contains estradiol and its metabolites (Johansen, 1985). Elevated levels of estradiol and testosterone are typical of fish undergoing gonadal growth, rather than spawning, so the use of these steroids as pheromones during final stages in reproduction is anomalous. The hormone directly associated with spawning in male teleosts is not clear, though a progesterone probably controls sperm hydration just prior to spawning (Fostier et al., 1987) and testosterone may influence male reproductive behaviour (Liley et al., 1987). The pheromone of the male African catfish is a progesterone (Resink, 1988); and the pheromones of the black goby (Colombo et al., 1980) and Baikal sculpin (Stacey and Sorensen, 1991) are androgens, so the few male pheromones identified may fit the proposed model.

Responsiveness to the pheromone also appears to be regulated by the reproductive state, although olfactory sensitivity in some species may not be. Most of the known pheromones induce a response only in fish of the appropriate reproductive state (Stacey and Sorensen, 1991; Van Weerd and Richter, 1991). However, Sorensen et al. (1988) and Resink (1988) have shown that high olfactory sensitivity for pheromones persists throughout the year in goldfish and African catfish, whereas in some other

cyprinids a seasonal change in sensitivity occurs (Sorensen et al., 1987). Also, Cardwell et al. (1995) have demonstrated that implanted androgens can selectively enhance olfactory sensitivity to a putative reproductive pheromone in male *Puntius schwanenfeldi.*

Pacific herring respond to the spawning pheromone only when they are producing milt or are ovulated and the testes contain pheromone only when fully mature (Stacey and Hourston, 1982). Thus, pheromone production and reception is limited to a particular reproductive state in this species. Elucidation of the hormonal control regulating reproduction in herring could both provide clues to the chemical nature of the pheromone and indicate how the reproductive state interacts with pheromonal communication in this species.

The hormones involved in the reproductive physiology of herring are unknown, though generalized models of reproductive endocrinology of fish have been proposed (Nagahama, 1994; Fostier et al., 1987). Information on the structure and function of reproductive hormones of the herring is of interest for predicting what hormones may be available for pheromonal communication and for investigating factors that may regulate responsiveness to the pheromone. In addition, this knowledge about a primitive teleost such as the herring contributes to our understanding of hormone evolution.

In this thesis, I characterize the structure and function of the spawning pheromone of Pacific herring and investigate the reproductive physiology underlying the pheromonal communication system. This was done by: 1) characterizing the response to the pheromone and factors regulating it, 2) characterizing the patterns of reproductive steroids found during reproduction in the herring, 3) investigating correlations between levels of steroidal hormones and responsiveness to the pheromone and

4) characterizing the structure of the spawning pheromone. This work is presented in Chapters 2-5. In addition, I furthered the development of the herring model for pheromonal communication by helping to elucidate the structures of gonadotropin releasing hormone and gonadotropin-II ß subunit present in this species of fish (Appendices A and B). These proteins have a central role in the control of reproduction in fish (Sherwood, et al., 1994; Querat, 1992; Swanson, 1992), but understanding of their function will be furthered by knowledge of their actions in a primitive teleost such as the herring. Structural elucidation is an important first step in pursuing this line of research.

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CHAPTER 2. BEHAVIORAL CHARACTERIZATION OF PHEROMONE-INDUCED SPAWNING OF PACIFIC HERRING[1]

ABSTRACT

A spawning pheromone in the milt (semen) and testes of the Pacific herring, *Clupea harengus pallasi*, triggers spawning in sexually mature fish of both sexes and is thought to facilitate school spawning of this species. I found the response to the pheromone to be a stereotyped behavioral sequence consisting of a graded extension of the gonadal papilla, release of gametes, and spawn deposition behavior. The response is triggered by an olfactory stimulus, as demonstrated by the elimination of the response by occlusion of the nares. Stimulus concentrations of approximately a 1:500 dilution of fresh milt or the equivalent of 0.02 g of fully mature testes/ml were required to elicit a response in 50% of ripe herring that are responsive to the pheromone. Female fish appeared to be less sensitive to the pheromone in milt than males early in the spawning season, but not thereafter. The average duration of responses of male fish was longer in response to concentrated milt than to testes extracts, but no consistent difference in response times between the two sexes was detected. Factors other than the spawning pheromone, maturity of the fish and stress were also found to influence the spawning response. For example, exposure to shallow (3 cm) water in a small tank induced "spontaneous" papilla extension and spawning approximately 20 min after refilling the tank; occluding the nares prevented this response. Also, the presence of floating kelp

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(Macrocystis) resulted in prolonged spawning in a large tank after a pheromonal stimulus.

INTRODUCTION

Pacific herring, *Clupea harengus pallasi*, are primitive teleosts that spawn in large schools in a *connubium confusum* (Breder and Rosen, 1966) considered ancestral to other teleost mating systems (Balon, 1975; Turner, 1986). School spawning such as this, without pair formation or physical interaction between sexes, is distinctive of this subspecies of herring (Schaeffer, 1937; Stacey and Hourston, 1982), although some controversy about this conclusion exists in the literature (Rounsefeld, 1930). Other subspecies of herring may spawn with greater interaction between sexes (Aneer et al., 1983; Ewart, 1884; Holliday, 1958). The school spawning of Pacific herring is apparently facilitated by a pheromone that is present in the milt of this species and induces spawning behaviour of sexually mature fish of both sexes (milt-producing males and ovulated females) (Stacey and Hourston, 1982; Sherwood et al., 1991; Carolsfeld et al., 1992). The spawning behaviour starts with the extension of the gonadal papilla and culminates in the deposition of trails of adhesive eggs or viscous milt (Stacey and Hourston, 1982). The pheromone is one of the clearest examples of a reproductive-releaser pheromone in fish in that the spawning behaviour is initiated within minutes of exposure. Attempts to induce large-scale spawning of captive herring in ocean pens with herring milt, however, have been largely unsuccessful (D. Gillis, pers. comm.; Kreiberg and Carolsfeld, unpub. obs.). The sexual maturity of the fish and the stress of the captive situation are the two most likely factors to influence responsiveness to the

pheromone. Fish appear to respond to reproductive pheromones only when at the appropriate level of maturity (Van Weerd and Richter, 1991) and, indeed, immature herring do not respond to the spawning pheromone (Stacey and Hourston, 1982). Stress is considered generally to be inhibitory to reproduction in fish (Sumpter et al., 1994). However, stress also may be stimulatory in that mature herring often spawn after being captured and/or crowded during commercial fishing, a response that is generally attributed to stress due to handling. Thus, there is a need to correlate reproductive maturity and stress level with differences in pheromone responsiveness among adult fish. However, the practical difficulty of working with herring has meant that information on reproductive hormones became available only recently (Carolsfeld et al., 1996; Chapter 3).

A procedure for maintaining mature captive Pacific herring in net pens for prolonged periods of time was developed in the 1980's (Kreiberg et al., 1982). Stacey and Hourston (1982) transferred such captive fish to smaller tanks for the initial description of the herring spawning pheromone. More recently, we developed an aquarium-based assay for screening pheromone-containing extracts, in which individual herring in small aquaria are exposed to a small bolus of pheromone-containing test solutions (Sherwood et al., 1991; Carolsfeld et al. 1992). Our results suggested that these captive herring and the pheromone assay could be useful tools for investigating the factors that regulate the response to the pheromone, once the behaviour under these conditions was adequately characterized. In this chapter, I investigate the response to the spawning pheromone and explore some of the factors that influence responsiveness to the pheromone in the aquarium assay and in larger tanks. In Chapter 4 I investigate the relationship between responsiveness to the pheromone

and plasma levels of steroid hormones indicative of levels of maturity and stress.

MATERIALS AND METHODS

Fish. Adult herring were captured by purse seine in Trincomali Channel, B.C. in mid-February prior to ovulation and milt production. These fish were 80-200 g in weight and 75-150 mm in length. The fish were transported by a live holding barge to the Pacific Biological Station in Departure Bay, Nanaimo, B.C., where they were maintained in 20 x 30 x 5m deep net pens without artificial feeding until after spawning had occurred in mid-May to early June. Stocking density was about 1 ton of fish per net pen. After the majority of fish had ovulated or commenced producing milt in early March, batches of 100-200 individuals of mixed sex were transferred to 1200 and 600 l circular holding tanks, 3 and 2m in diameter, where they were held up to 1 month. These tanks were supplied with 11-13 °C flowing sea water pumped from Departure Bay. Fish were netted at random from the holding tanks, sexed by the presence of milt or eggs expressed with gentle abdominal pressure, and transferred in lots of 25-30 fish of a single sex to darkened 60 l holding buckets with running sea water. Fish were held in these holding buckets for up to 3 hours before they were netted and transferred to assay aquaria for the various experiments described below. Similar fish were used for experiments in larger tanks at the Bamfield Marine Station (Bamfield, B.C., Canada), but these herring were captured in Barkley Sound in the vicinity of Bamfield. The fish were maintained in a 10m diameter concrete tank of 3m depth at the Bamfield Marine Station rather than in net pens, and were fed frozen zooplankton *ad libitum.*

Pheromonal materials. Fresh milt, supernatant from centrifuged fresh milt, or testes extracts were used as pheromonal stimuli, as indicated in the various experiments. Milt was collected from fish in the holding tanks throughout the spawning season by stripping ripe male fish with gentle abdominal pressure, whereas testes were taken from freshly killed ripe fish from the net pens early in the spawning season. I found that pheromonal activity in extracts of milt and testes was present in high pressure liquid chromatography fractions that eluted in similar positions, suggesting a comparable chemical composition (results not shown). Milt supernatant was prepared by centrifuging freshly expressed milt for 15 minutes at 4°C and 2500 x g. The milt pellet was rinsed twice with volumes of sea water equivalent to the original volume of milt. The three supernatant fractions were combined for use, either directly or diluted further with sea water. Testes extracts were prepared as described previously (Sherwood et al., 1991), except that the testes extracts used for threshold determination and the large tank experiment were eluted from a 2.5 x 16 cm bulk-packed C_{18} column with 80% acetonitrile, rather than from C_{18} Sep-Paks (both C_{18} materials were obtained from Waters Associates, Milford, MA). The concentration of each extract or milt preparation was calculated based on the original weight of the testes or volume of milt, i.e. 0.1 gram equivalent (g.e.)/ml indicates that 1 ml of the solution contains the extract of 0.1 g of testes or 0.1 ml of milt. These materials were diluted with distilled water or clean sea water, respectively, just prior to use. A single batch of milt or testes extract was used within each experiment unless otherwise specified.

SPECIFIC METHODS AND RESULTS

A) CHARACTERIZATION OF THE PHEROMONE RESPONSE:

1) DOCUMENTATION OF COMPONENTS OF THE PHEROMONE RESPONSE

Stacey and Hourston (1982) described the response to spawning pheromones by small schools of herring in tanks. The aquaria I used in my bioassays were a considerably different environment to that used by Stacey and Hourston (1982), so it was necessary to document the behaviour under the aquarium conditions before using the assay in characterizing factors influencing the response to the pheromone.

Methods

The response to the spawning pheromone by isolated fish was observed in conditions we described previously (Sherwood et al., 1991). Briefly, 1 m x 30 cm aquaria were filled to a depth of 25 cm (approximately 60 litres) with fresh sea water. Seven to ten aquaria were used in parallel, each with a single fish. Each aquarium was lighted from the top with a 25 watt incandescent light, covered with a black plastic sheet, and observed from a darkened room. Fish were placed into the aquaria and allowed to acclimatize for 20 minutes. Pheromonal stimulation was then carried out by applying a 0.25 ml bolus of milt or testes extract onto or immediately in front of the nares of the fish with a pipettor. The sequence of behavioural events that occurred in response to pheromonal stimulation was observed in about 1500 trials over

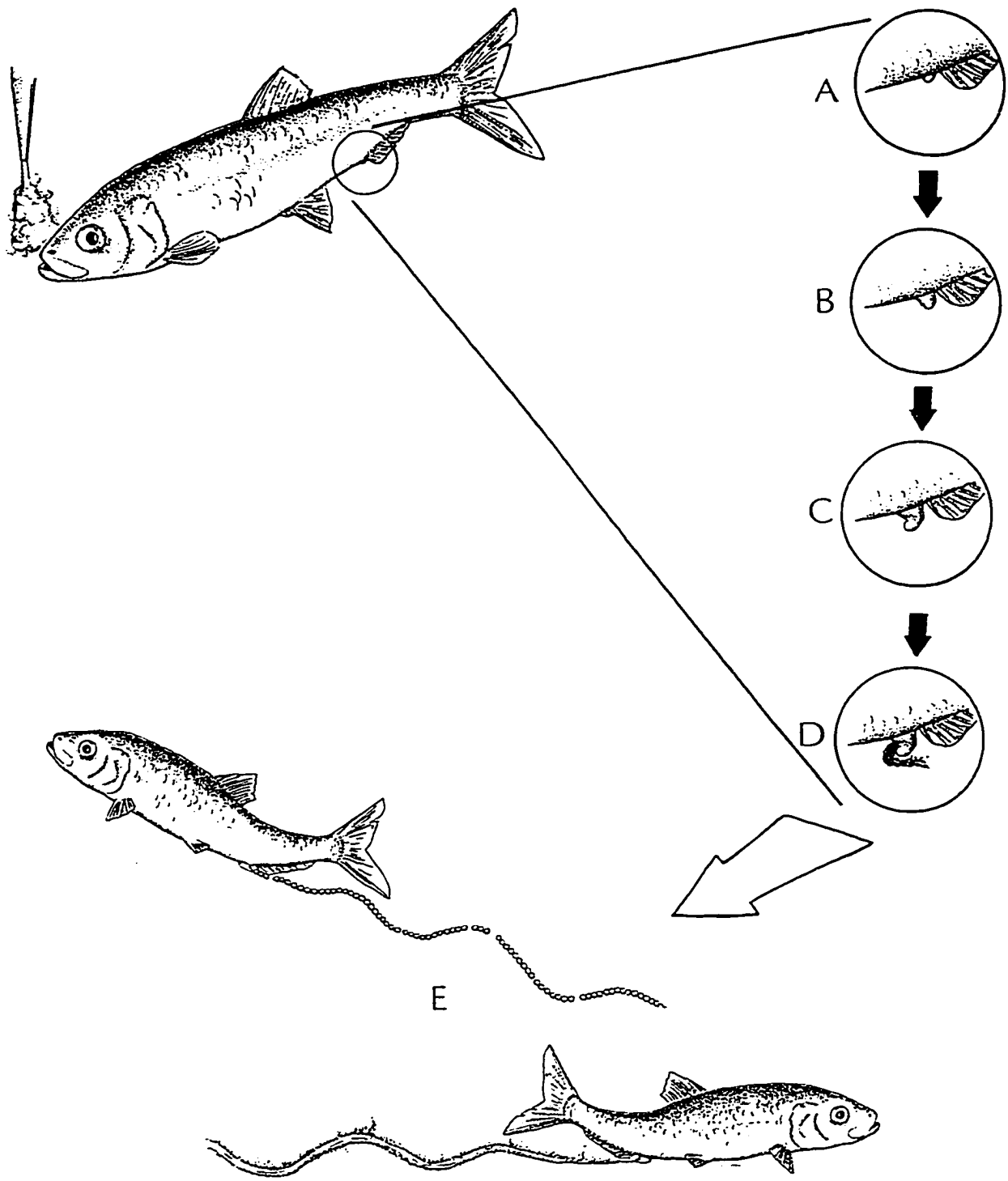
a period of three years. These form the basis of the descriptive summary presented in the results.

Results

The behavioural response of herring to a pheromonal stimulus in the assay aquaria (Fig. 1) consisted sequentially of: 1) extension of the gonadal papilla, 2) initiation of release of gametes ("mid-water spawning"), and 3) deposition of gametes onto a substrate with a distinctive spawning behaviour ("substrate spawning"). Exceptions to this sequence included the fairly common omission of mid-water spawning and rare occasions of initiated substrate spawning behaviour before papilla extension. Rapid swimming with the chin and pectoral fins touching the substrate was observed in the aquarium, but was independent of the addition of spawning pheromone(s).

The response to the pheromone was graded in that the sequence of events was not always fully expressed and in that intermediate levels of papilla extension were observed (recorded as slight + or medium+). To ensure that observations of the response were comparable between observers, a qualitative scale of papilla extension was used that was based primarily on the relative length and curvature of the papilla (beyond a medium papilla extension, the papilla becomes progressively more curved, to the point of a marked terminal hook in a full papilla extension; Fig. 1). The minimal response that I could detect was a change in the extension of the papilla that did not necessarily result in a fully extended papilla ("partial" papilla extension). However, transient partial extensions of the papilla were also observed to occur independent of overt stimulation and only the full papilla extension and spawning were found to be responses that could be recorded unambiguously by different observers. I thus included standardized observations of partial responses in descriptive work, but only considered full

Fig. 1: Components of the response to spawning pheromone in mature herring. A-C: extension of gonadal papilla from initially "slight" condition (A), to "medium" (B), and finally "full" (C) condition; D: midwater spawning; E: substrate spawning. Graded responses occur, with only partial extension of the papilla and intermediate stages of papilla extension (slight + and medium +). Substrate spawning without the intermediate step of mid-water spawning was also common. Only a full papilla extension or spawning were considered to be clearly positive responses to the pheromone.


A
B
C
D
E

papilla extension and spawning as positive responses to the pheromone in analytical experiments.

2) PATTERN OF PHEROMONAL EXPOSURE REQUIRED FOR ELICITING A RESPONSE

Herring in the assay aquaria are exposed to the pheromone not only with the first application of stimulation, but also with the progressively more dilute stimulus throughout a test. I thought it was important, therefore, to determine whether the concentration of stimulus at the first moment of exposure or the final concentration of pheromone in the tank was the critical factor in eliciting a response. To investigate this problem, I applied stimuli with an equal amount of pheromone in different initial stimulus volumes.

Methods

A stimulus of 0.02 gram equivalents (g.e.) of milt supernatant diluted in boli of 0.25, 0.5 and 1 ml volumes was used for this experiment. A 0.02 g.e. stimulus of milt supernatant in a 0.25 ml volume was previously determined to be slightly greater than the threshold stimulus for eliciting a full papilla extension. Each volume was tested separately on individual milt-producing fish (n=7) in the assay aquaria described above. Twenty minutes after the pheromonal exposures, fish were tested for pheromonal responsiveness with a 0.25 ml bolus of 0.5 g.e./ml of fresh milt (a standard suprathreshold stimulus), and those fish not responding were eliminated from the analysis. Results were compared with Fisher's exact probability test (Sokal and Rohlf, 1969) and assessed with an error rate (α) of 0.05.

To indicate the rate of diffusion of pheromonal stimuli applied in the manner described, I pipetted a 0.25 ml bolus of fluorescein dye into the aquaria with a single fish in an identical manner as described above. The dye was observed with the assistance of ultraviolet light, as well as being videotaped for later analysis. Relative concentration of dye was estimated by measuring the diameter of the observed cloud of dye, and comparing the cube of this value. This experiment was carried out three times.

Results

The 0.25 ml bolus of milt supernatant elicited a positive response in 6/7 fish, whereas the equivalent total amount of milt stimulus presented as a 0.5 ml bolus produced a positive response in only 2/6 fish and, when presented as a 1 ml bolus, activated only 1/5 fish. The responses to the 0.25 ml and the 1 ml stimuli were significantly different from each other, but not from the response to the 0.5 ml stimulus. Comparable stimuli of sea water alone failed to elicit a behavioural response.

The bolus of flouroscein dye administered in the manner of a 0.25 ml pheromonal stimulus first formed an irregular cloud of about 0.5 ml in size in the aquarium, and then dissipated throughout the aquarium within 1 to 1.5 minutes. Thus, a fish would be initially exposed to one half or more of the full concentration of the stimulus as applied from the pipettor, and would then swim through a progressively more dilute cloud of the stimulus three or four times before the material was fully dispersed in the aquarium and diluted by about 5×10^6 in 60 litre aquaria.

3) EFFECTS OF SEQUENTIAL PHEROMONAL STIMULI

The dye study (see above) suggested that pheromonal stimulation consists of a transient stimulus of suprathreshold concentration. The 0.25 ml milt stimulus of this experiment was diluted four-fold to a sub-threshold concentration in less than 1 minute. The fish swim through any given spot in aquaria of this size approximately every 40 seconds, so the response to the pheromone is most likely to result from exposure to the pheromone of less than 1 minute, rather than a prolonged exposure to dilute pheromone. Thus, I investigated how the response may vary with repeated stimulation in the assay aquarium. This scenario in some ways simulates situations encountered in a natural spawning, since the distribution of milt in the water is very patchy at the start of such a spawning. I used ovulated females for this experiment to avoid interference from milt that could be produced by males during such an experiment.

Methods

Ten ovulated fish housed in individual assay aquaria were exposed sequentially to a number of 0.25 ml stimuli of testes extract (Fig. 2). The initial two stimuli of 0.1 g.e./ml were spaced by ten minutes to test for priming for subsequent responses (no evidence of a delayed response to subthreshold stimuli was found in related trials as described in the next section). This test was repeated in twenty minutes using two stimuli of 1 g.e./ml. Subsequently, stimuli of 1 g.e./ml were applied for three hours, each following the previous one by twenty minutes. The twenty minute time period appeared to allow for complete recovery from most responses. After three

hours, the concentration of the stimuli was increased first to 10 g.e./ml and then 100 g.e./ml, still with 20 minute periods between stimuli. Fish were observed continuously and changes were noted when they occurred. Dissolved oxygen and water temperature in the assay aquaria were monitored during several of these trials with an Oxyguard portable oxygen probe (Zeigler Bros. Inc., Gardners, PA.) and a hand-held bulb thermometer, respectively.

Results

Representative response patterns of ovulated fish to sequential pheromonal stimulation are shown in Fig. 2. Fish A was a "responsive" fish, demonstrating that: 1) sensitization by a subthreshold stimulus primes the fish for a positive response to the same stimulus presented within 10 minutes; 2) full recovery from a response occurs within 20 minutes, after which further comparable responses can be elicited without evidence of marked facilitation; 3) immediate refractoriness to stimulation is not evident, although long-term acclimatization is, as responses are reduced after 2 hours in the assay aquarium; 4) once responses are reduced, full responses are elicited again with an increased stimulus strength. Fish B was a "non-responsive" fish that showed no response to the pheromone. Fish C was a "spontaneous" fish that showed initial papilla extension without overt pheromonal stimulation, but also responded to extract and showed priming with the lowest concentration of pheromone. Spontaneous fish that showed no response to pheromonal stimulation also were encountered (not shown). No evidence was found for responses that were delayed longer than 3 minutes in onset.

Average dissolved oxygen in aquaria of individual assays was reduced from 9.5 ppm to 7.5 ± 0.6 ppm during these sequential trials, whereas water

Fig. 2: Representative responses of ovulated herring to sequential pheromonal stimulation in individual assays. See text for assay details. A, responsive fish; B, non-responsive fish; C, spontaneous fish responsive to pheromone. Arrow heads under traces indicate times of stimulus application, and are the same for A-C. Stimuli for A-C consisted of testes extracts of 0.1, 1, 10, and 100 g.e./ml. (g.e.= gram equivalents of tissue, milt or testes, used for preparation of pheromonal material). Papilla condition: Sl=slight papilla, M=medium papilla, F=full papilla extension (see Fig. 1 for illustration). Intermediate levels of papilla extension (Sl+, M+) were also recorded.

Papilla condition
F
M
Sl
A
B
C
100
10
1
0.1
Stimulus strength
(g.e./ml)
0
30
60
90
120
150
180
210
240
Time (min)

temperature increased from 10 °C to 14.5 ± 0.2 °C (n=10). Behaviors indicative of oxygen or temperature stress were not generally observed.

4) THRESHOLD CONCENTRATIONS OF PHEROMONE FOR RESPONSES

Determination of the concentration of a pheromone needed to elicit responses would ideally be done with the pure or synthetic form of the pheromone. However, until the structure(s) is isolated and identified it is important to standardize the biological preparations containing pheromonal activity and to characterize the response of the bioassay animal for differences due to gender or reproductive maturity. I thus carried out some preliminary experiments to test the responsiveness of male and female fish to fresh milt or a standardized testes extract early and late in the spawning season. The response to fresh milt would provide an indication of the combined effect of changes in pheromonal content in the milt and responsiveness to the pheromone, whereas the response to the testes extract would provide an indication of changes in responsiveness to the pheromone alone.

Methods

The concentration of fresh milt or testes extract required to elicit a positive response (full papilla extension and/or spawning) was determined for males and females early and late in the season. The herring in our holding facility become fully mature (ovulated and milt-producing) in mid-March and can remain overtly unchanged until spawning up to two months later in May. I sampled fish in late March/early April ("early" sampling period) and early

May ("late" sampling period). Determinations were carried out in ten individual assay aquaria for each sex, using fish transferred directly from the holding tanks. Increasing concentrations of pheromonal stimuli were applied sequentially to each fish at 20 minute intervals, in the case of testes extract stimuli at concentrations of 0.01, 0.1, 1, and 10 g.e./ml., and in the case of milt stimuli at concentrations of 0.001, 0.01, 0.1 and 0.5 g.e./ml, followed by a stimulus of 10 g.e./ml of testes extract. Milt was harvested from 10-15 fish on the day of testing and combined. This pool of milt was used for all fish within the sampling date. In contrast, aliquots of a standard testes extract were used on all fish both early and late in the season. This testes extract was stored frozen until use, a storage procedure found earlier to maintain pheromonal activity for over two years. Fish were classified according to the lowest concentration of pheromonal substance that elicited a positive response (full papilla extension and/or spawning). Individuals that showed a full papilla extension or spawning without overt pheromonal stimulation were classified as "spontaneous", whereas fish that did not respond to the maximal dose tested were classified as "non-responsive".

The results of these experiments were plotted as cumulative proportions of responsive fish in the total number of fish. Values at each concentration were compared with each other using tabulated 95% binomial confidence limits (Tate and Clelland, 1957). The "effective" concentration (ED_{50}) of milt or testes extract required for a response was defined as the concentration needed to elicit a positive response in 50% of the fish "responsive" to the pheromone. The data from the response curves that were not significantly different from each other were pooled within each stimulus type for the ED_{50} estimate to increase the effective sample size. These composite response curves were also compared for significant differences as described above (Fig. 3A, B).

The ED_{50} of milt required to elicit a positive response in fish responsive to milt was between 0.001-0.01 g.e./ml, whereas the comparable value for the testes extract was between 0.01-0.1 g.e./ml. The response curves to the two types of stimulus were also significantly different for the 0.01 and 0.1 g.e./ml concentrations (Fig. 3C).

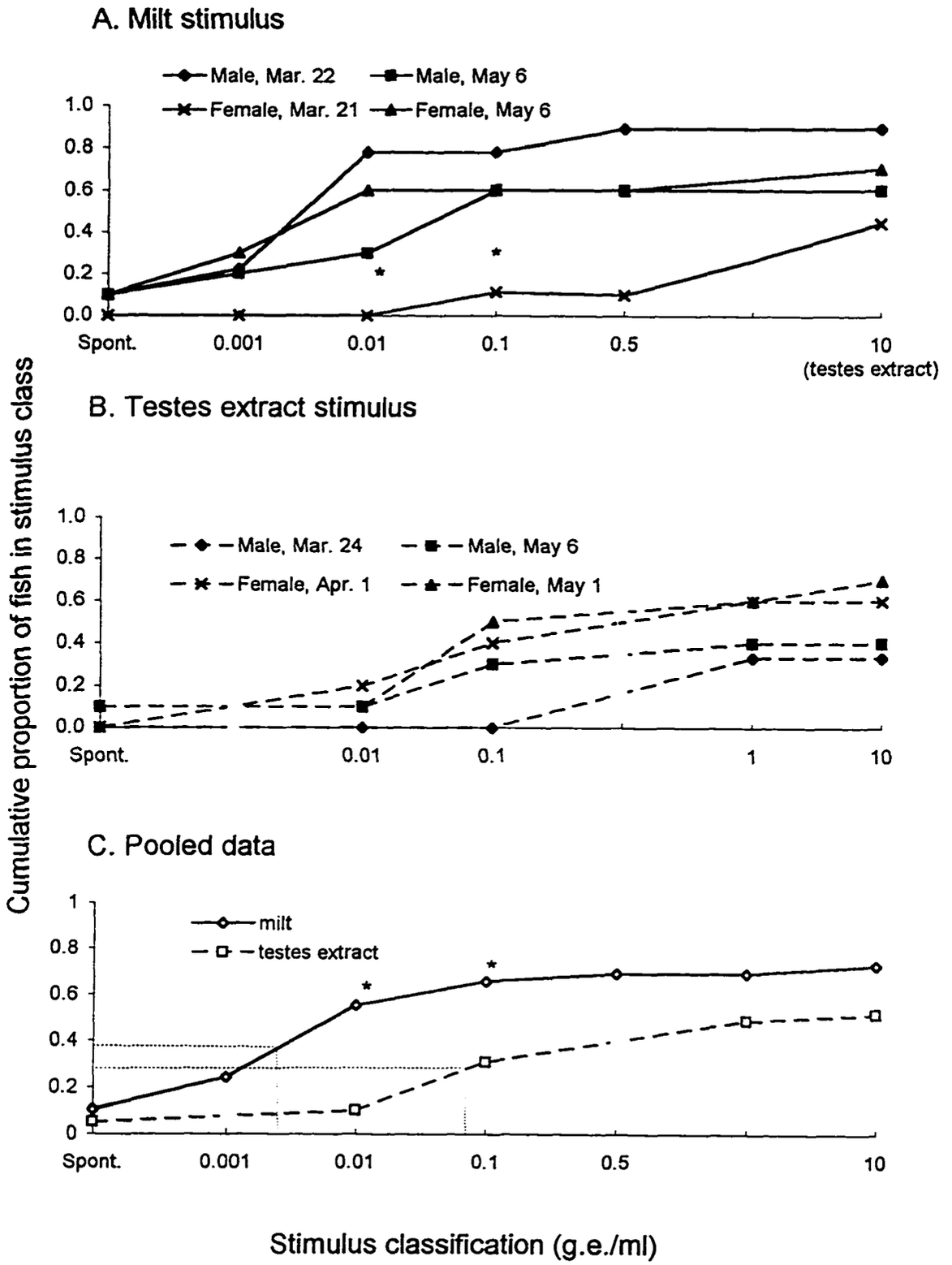
5) SENSORY PATH INVOLVED IN PHEROMONAL RECEPTION

Pheromone detection in fish appears to occur primarily through olfaction (see Sorensen, 1992), but several other chemosensory mechanisms exist in the typical fish, including gustation and a "common chemical sense" (see Hara, 1992). I thus tested whether the spawning pheromone of herring was detected by olfaction.

Methods

The nares of seven milt-producing fish were occluded with a drop of Vetbond tissue adhesive (3M, St. Paul, MN) immediately after confirmation of their responsiveness to the pheromone with the group assays described below for the experiment on individual consistency of response, but using a 1 g.e./ml testes extract as screening stimulus. A further five responsive fish were treated as controls with a drop of cement adjacent to the nares. The fish were identified by fin clips and housed together in a 600 litre tank. Five days later they were re-tested for responsiveness to the pheromone in assays of individual fish with 0.25 ml of a 10 g.e./ml testes extract. Pheromone responsivity (presence or absence of a full papilla or spawning

Fig. 3: Classification of herring by concentration of A) milt or B) testes extract required to elicit a positive response (n=10 per sample). Asterisks (*) indicate significant difference from the March 22 male sample as determined by binomial error intervals (α=0.05). C) Estimation of "threshold" concentration of milt or testes extract needed for a positive response in 50% of "responsive" fish, based on pooled data of A) and B) except for early season female fish of the milt stimulus group. Asterisks (*) indicate significant differences as determined by binomial error intervals (α=0.05).


A. Milt stimulus
Male, Mar. 22
Male, May 6
Female, Mar. 21
Female, May 6
1.0
0.8
0.6
0.4
0.2
0.0
Spont.
0.001
0.01
0.1
0.5
10
(testes extract)
B. Testes extract stimulus
Male, Mar. 24
Male, May 6
Female, Apr. 1
Female, May 1
Spont.
0.01
0.1
1
10
C. Pooled data
milt
testes extract
1
0.8
0.6
0.4
0.2
0
Spont.
0.001
0.01
0.1
0.5
10
Cumulative proportion of fish in stimulus class
Stimulus classification (g.e./ml)

response) of the treatment groups was compared with pair-wise Fisher's exact probability tests (Sokal and Rohlf, 1969), assessed with an error rate (α) of 0.05.

Results

Fish with both nares blocked by tissue cement no longer responded to pheromonal stimulation (0/7), whereas 5/5 of the control fish were still responsive. The responses of the occlusion and control groups were significantly different in this experiment.

B) FACTORS INFLUENCING THE RESPONSE TO THE SPAWNING PHEROMONE:

1) FACTORS INFLUENCING THE TIME COURSE OF THE PHEROMONAL RESPONSE

The time course of the response to the pheromone is important for description of the behaviour and for determining how stereotyped the behavioural pattern is. The influence of factors such as sex, stimulus strength, and levels of excitation on the time course can also indicate how important these factors are in the regulation of the response.

Methods

I recorded response times to a pheromonal stimulus in 106 individual assays during late March and early April, using both ripe male and female

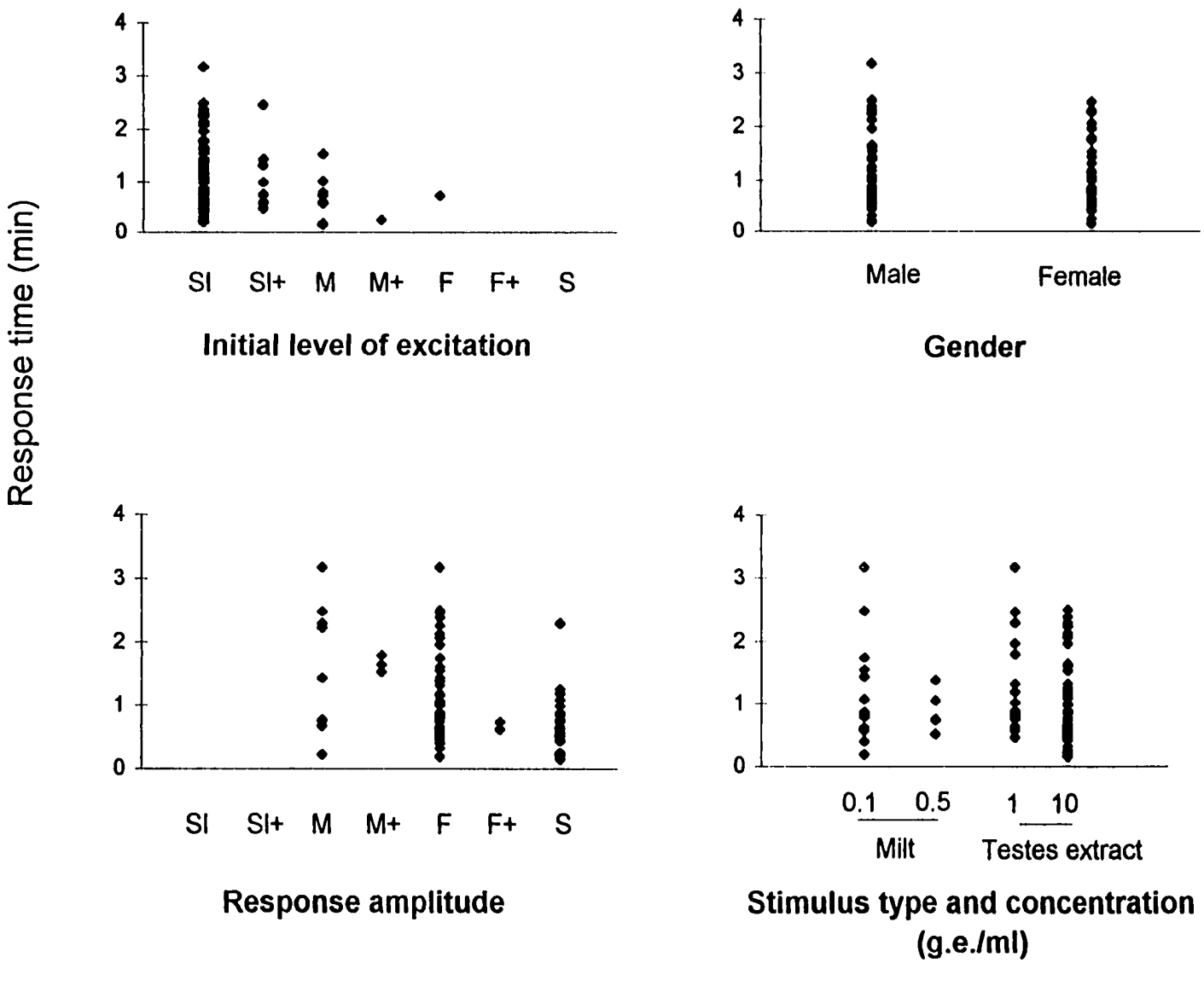
fish. Stimuli consisted of fresh milt of 0.1 or 0.5 g.e./ml or testes extract of 1 or 10 g.e./ml. "Response" time is the time from initial exposure to the stimulus to the maximal expression of the response. Fish were observed continuously for this period of time. "Recovery" time is the time from maximal expression of the response to the retraction of the papilla to its initial state. Observations were made every 2-3 minutes. Recovery times were recorded in 67 of the assays used for measurement of response times. The influence of sex; type and concentration of pheromonal stimulus; initial level of excitation; and response amplitude on response and recovery times, as well as the correlation between response and recovery times, was investigated with multiple regression and ANOVA analyses with an error rate (α) of 0.05 (Microsoft, 1994).

"Latency" time is defined as the time from stimulation to the first visible sign of a response. Latency was measured in 29 of the assays. Latency times were assessed from fish observed continuously during their response to the pheromone. The relationships between latency and recovery times were assessed with simple linear regressions (Microsoft, 1994). All values are presented as means ± standard errors.

Results

Response time (from pheromonal stimulation to maximum response) varied from 0.2 to 3.2 min, with an average of 1.1 ± 0.1 min (n=106). This time appeared influenced by the amplitude of the response attained and the initial level of excitation in the multiple regression, but appeared independent of gender, stimulus type and stimulus concentration. However, none of these trends were markedly evident in scatter plots of the data (Fig. 4), and none remained as a significant influence in the subsequent univariate

Fig. 4: Scatter plots of response times and factors potentially influencing the response to the spawning pheromone. Levels of excitation and response amplitudes considered are as outlined in Figure 1, with the addition of F+ to denote exceptionally pronounced papilla extensions.


Response time (min)
4
3
2
1
0
SI
SI+
M
M+
F
F+
S
Initial level of excitation
Male
Female
Gender
Response amplitude
0.1
0.5
1
10
Milt
Testes extract
Stimulus type and concentration (g.e./ml)

ANOVAs that avoid assumptions of linearity and the influence of unequal sample sizes. (Table 3). Latency time (from pheromonal stimulation to initiation of response) varied from 0.1-2.6 min, and averaged 0.8 ± 0.1 min (n=29), associated with a mean response time of 1.6 ± 0.2 min in this sample. The two measures were positively correlated (r^2 = 0.68, P <0.0001). Latency time and recovery times were not significantly correlated in this sample (r^2 = 0.01, P = 0.67). The proportion of female fish was slightly greater in the sub-sample of responses for which latency measures were obtained than in the overall group of assays (66% compared with 51%), but the representation of stimulus types and concentration was proportional to the overall group

Recovery time from a pheromonal stimulus (measured from the time of maximal response to the full retraction of the papilla) varied from 0.2 to 29.3 min, with an average of 11.2 ± 0.9 min. (n = 67). This measure was significantly influenced by stimulus type and concentration and the gender of the fish in the multivariate analysis, but not by other factors studied (Table 3). A closer analysis of these factors (Table 4) indicated that the 0.5 g.e./ml milt stimulus resulted in the most prolonged responses, particularly in male fish, but a consistent difference between sexes was not evident. Milt was released by some of the male fish in the assays used for determining recovery time (7/22 males), but mean recovery time of this subset was not significantly different from that of the other males.

2) INDIVIDUAL CONSISTENCY OF RESPONSIVENESS TO PHEROMONE

The previous two sections indicate considerable variation in the sensitivity of individual fish to the spawning pheromone, including the absence of a response in some fish to even the most highly concentrated I

Table 3. Evaluation of influence of test factors on response and recovery times of the spawning pheromone response

Temporal Element	Test Factor	Multiple Regression[1] R^2	Multiple Regression[1] P	Single factor ANOVA[2] P	Single factor ANOVA[2] df	total df
Response time						
	multiple comparison	0.21	0.000			105
	initial level of excitation		0.001	0.370	4	
	response amplitude		0.001	0.185	4	
	sex		0.582			
	stimulus concentration		0.285			
	stimulus material		0.334			
Recovery time						
	multiple comparison	0.33	0.000			66
	initial level of excitation		0.24			
	response amplitude		0.07	0.145	5	
	response time		0.08	0.121	1	
	sex		0.00	0.036	1	
	stimulus concentration		0.60			
	stimulus material		0.00	0.000	4	

[1] multiple regression between temporal value and numerically coded test factors

[2] single factor ANOVA treating levels of test factors and combinations of test factor levels as separate levels of the ANOVA

Table 4. Influence of stimulus type and concentration on response and recovery times of pheromonal response

Material	Conc. (g.e./ml)	Gender[1]	Response time[2] (min.)	Recovery time[2] (min.)	n response	n recovery
Fresh milt	0.1	m	1.77 ± 0.51	5.54 ± 0.67 [a,c]	5	3
		f	1.75 ± 0.53	5.12 ± 4.97 [a,c]	4	2
	0.5	m	0.89 ± 0.15	26.24 ± 1.05 [b]	5	5
		f	1.36 ± 0.42	16.7 ± 5.14 [a,b]	5	5
Testes extract	0.1	m	n/a ± n/a	n/a ± n/a	0	0
		f	0.91 ± 0.13	6.65 ± 2.35 [a]	10	8
	1.0	m	1.13 ± 0.26	7.78 ± 0.5 [a,c]	10	7
		f	1.47 ± 0.28	n/a ± n/a	7	0
	10	m	0.93 ± 0.12	4.01 ± 0.61 [c]	32	14
		f	0.86 ± 0.1	12.74 ± 1.03 [a]	28	23

[1] m = male; f = female

[2] means ± standard error; different superscript letters denote significant differences as determined by Tukey's multiple comparison ($\alpha = 0.05$)

stimulus tested. A necessary precedent to studying this variation is a knowledge of how consistent the responsiveness to the pheromone is in individual fish. To investigate this, I sorted a sample of fish as responsive and non-responsive to the pheromone and, after a period of holding in the laboratory tanks, re-tested the same fish.

Methods

I sorted milt-producing fish as "responsive" and "non-responsive" with "group assays". In these assays, groups of five randomly selected milt-producing herring were placed in 30 l glass aquaria lighted from the top with a shrouded 25 watt light. The fish were observed for a 15 minute acclimatization period from a darkened room. "Spontaneous" fish that showed prolonged extension of the gonadal papilla or showed indications of substrate spawning behaviour during this period were carefully removed by hand, prior to milt release, and eliminated from the experiment. After the acclimatization period, 1 ml of pheromonal stimulus (fresh milt diluted 1:1 with sea water) was applied to the center of the aquarium by pipette. This stimulus was repeated twice, once after 10 seconds and once after 2 minutes. Fish that responded positively to the milt by extension of the gonadal papilla were classified as "responders", while those that did not were classified as "non-responders". Individual fish in this size of assay aquarium swam through the center of the aquarium approximately every 5-10 seconds, so I felt they were very likely to encounter a pheromonal stimulus of adequate concentration to permit reliable classification.

15 responsive and 40 non-responsive fish were sorted into five separate 60-litre covered holding buckets and were maintained in these

buckets for 2 hours. All responsive fish were placed together in one bucket, whereas non-responsive fish were placed in groups of 10 fish into the other four buckets. The bucket of initially responsive fish and one of the buckets of non-responsive fish were supplied with a flow of sea-water of 5 l/min, one of the other buckets of non-responsive fish was supplied with 2.5 l/min of sea water, and the remaining two buckets of non-responsive fish were left without water flow. Ten ml of fresh milt was added to one of the holding buckets without water flow, prior to the introduction of fish. After the holding period, pheromone responsiveness of the fish was reassessed with the same procedure as used initially. Changes in responsiveness were evaluated with Fisher's exact probability tests (Sokal and Rohlf, 1969).

Results

The tendency to spawn spontaneously and responsiveness to the pheromone changed in some fish in this experiment. After holding, 6/40 of the initially non-responsive fish became responsive (1/10 in each of three buckets, and 3/10 in the bucket with added milt), whereas 10/15 of the initially responsive fish became non-responsive, 3/15 spawned spontaneously, and 2/15 were still responsive to the pheromone. Both the loss of responsiveness (10/15 fish) and the total gain in responsiveness (6/40) correspond to statistically significant changes relative to the original responsiveness to the pheromone (α=0.05). This experiment was not adequate to determine causes for changes in responsiveness to the pheromone, but does indicate that this is a character that can be either lost or gained over a relatively short period of time.

3) FACTORS INFLUENCING RESPONSIVENESS TO THE SPAWNING PHEROMONE

As indicated in earlier sections, a significant proportion of fully mature herring do not respond to the spawning pheromone in our assays. The experiment in the last section, although not adequate to determine causes for changes in responsiveness to the pheromone, indicated that this is a character that can be either lost or gained over a relatively short period of time. Some of the factors that regulate reponsiveness to the pheromone in captivity may also be important in regulation of natural spawning, so are of considerable interest. In a parallel project that is carried out throughout the spawning season (Chapter 6), I screened fish for pheromonal responsiveness before using them in assays for guiding purification of the pheromone. I have hypothesised that subtle differences in sexual maturity and stress are two of the most likely factors to influence responsiveness to the pheromone, and felt that this screening data provided an opportunity to investigate this hypothesis. Differences in sexual maturity are represented in this screening data by assay date (i.e. progress in the spawning season), whereas differences in stress are represented by time spent in the relatively large holding tanks of the compound and time spent in the relatively small holding buckets of the laboratory. In addition, the screening data allows an investigation of diurnal differences in responsiveness to the pheromone and differences in the proportion of fish that spawn spontaneously in the assay aquaria.

Methods

Pheromonal responsiveness was assessed with "group assays", described above for the experiment on consistency of pheromonal responsiveness, but using a single, rather than repeated, 1 ml pheromonal stimulus. These assays permit a more efficient screening of fish for pheromonal responsiveness than individual assays, but are relatively reliable (see earlier discussion in experiment on consistency of response).

Pheromonal responsiveness of milt-producing herring was determined from early-March to early-May at all times of the day and night. The fish were kept in 3 different tanks for 0-26 days prior to transfer to the lab, and were then held in the lab from 0-8 hrs. The proportion of spontaneous fish in a total of 126 screening assays was analysed, whereas only screening assays in which milt (0.5 g.e./ml) was used as the stimulus were considered for the analysis of the proportion of responsive fish (n=64).

The relationship between the proportions of responsive and spontaneous fish in the screening assays and the above factors was investigated with multiple regression followed by bivariate correlation analysis of factors contributing at a level of $P < 0.1$, as described for the analysis of response and recovery times above. Proportions were arcsine transformed before analysis to enhance normality of the data (Sokal and Rohlf, 1969).

Results

The proportion of spontaneous fish in the screening assays was positively correlated with holding time in the tanks and negatively correlated with the proportion of responsive fish (Table 5; Fig. 5). The proportion of responsive fish in the screening assays was negatively correlated with the date of screening and the proportion of spontaneous fish (Table 5, Fig. 6). The multiple correlation coefficient (R^2) of the regressions of both groups of

fish were small, as were the univariate correlation coefficients (r^2) of factors found to be significant. Thus, the factors I investigated explained only a small proportion of the variability in the occurence of spontaneous and responsive fish in the screening assays.

4) INDUCED SPAWNING BY BRIEF EXPOSURE TO SHALLOW WATER

Oral tradition of herring fishermen suggests that herring spawning can be induced by lifting the bottom of the net enclosure and exposing the fish to the air or shallow water for a period of time. Natural spawning of the Pacific herring also occurs in quite shallow water, and the fish are believed to swim rapidly over the spawning ground prior to initiating spawning (Hay, 1985). I drained the water to a low depth in 2m diameter tanks containing milt-producing fish late in the season of 1996 to investigate the effect of this treatment on herring behaviour. The herringspawned in this preliminary trial, but only after the tank had been refilled (45 minutes after initial exposure to the shallow water). In contrast, the spawning response in assay aquaria was initiated within 3 min of pheromone exposure. Thus, we felt that the shallow water-induced spawning may be triggered by a factor such as stress, independent of the spawning pheromone that may be squeezed out of the fish during shallow water exposure. I thus investigated the effect of occluding the nares of the fish during the treatment, reasoning that a stress-induced response should not be dependent on olfactory cues. The control group in this experiment had one naris occluded to correct for any stressful influence of the occlusion process.

Table 5. Influence of test factors on proportion of spontaneous and pheromone-responsive fish during group assays[1]

Pheromone responsivity	Test Factor	Multiple Regression R^2	Multiple Regression P	Single factor comparison P	Single factor comparison r^2	total df
Spontaneous						
	multiple comparison	0.17	0.00			127
	date of screening		0.51			
	holding time in compound		0.02	< 0.05	0.04	126
	holding time in lab		0.49			
	proportion of responsive fish		0.00	< 0.05	0.06	126
	source tank		0.42			
	time of screening		0.28			
Responsive[2]						
	multiple comparison	0.18	0.08			63
	date of screening		0.07	0.05	0.07	62
	holding time in compound		0.84			
	holding time in lab		0.87			
	proportion of spontaneous fish		0.10	< 0.05	0.13	62
	source tank		0.94			
	time of screening		0.86			

[1] comparison of arcsine transformed proportions (ripe male fish only).

[2] groups assays with 0.5 g.e./ml milt stimulus

Fig. 5. Proportion of spontaneous fish encountered in screening assays plotted against individual variables considered as possible influencing factors.

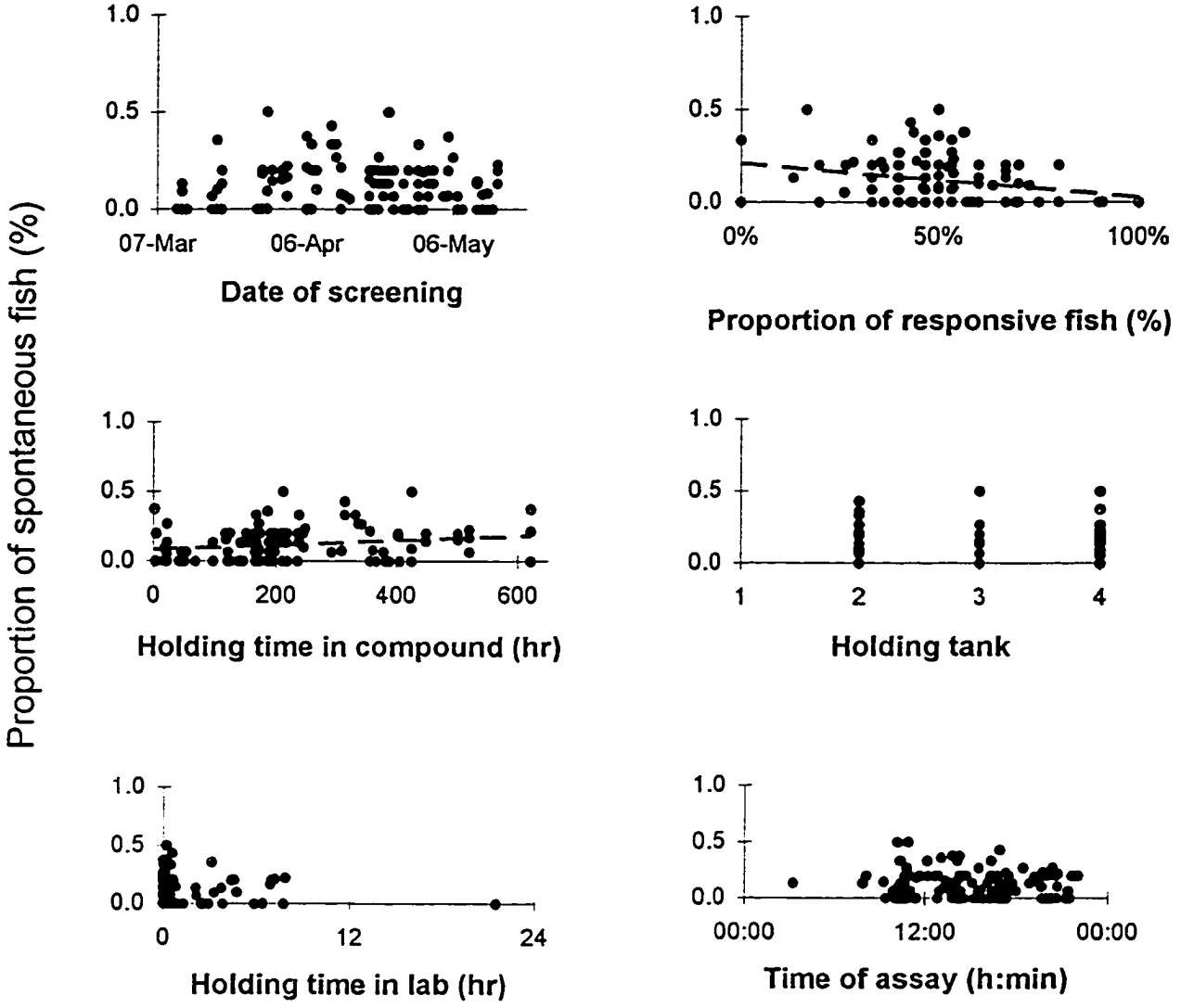
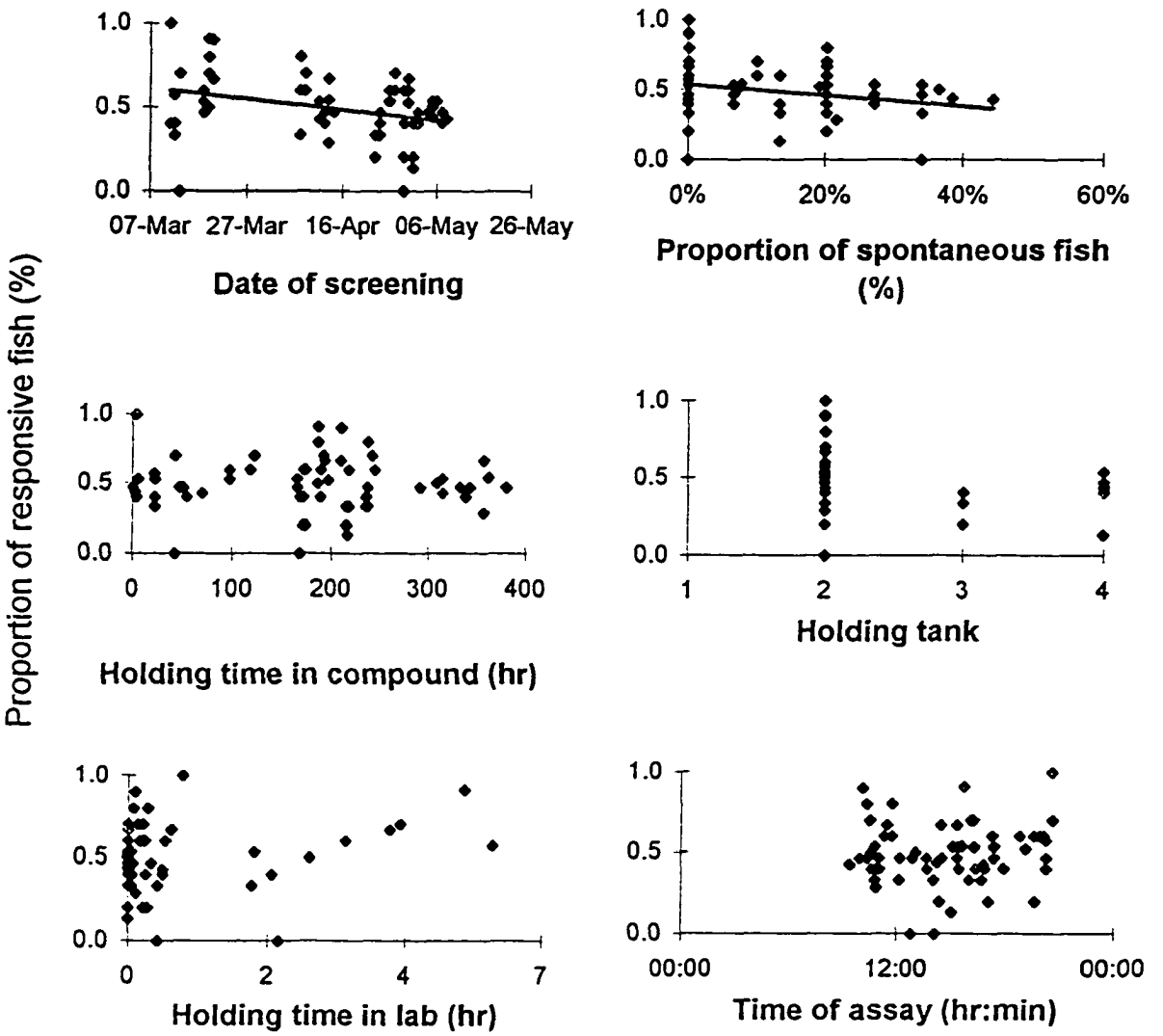

Proportion of spontaneous fish (%)
1.0
0.5
0.0
07-Mar
06-Apr
06-May
Date of screening
0%
50%
100%
Proportion of responsive fish (%)
0
200
400
600
Holding time in compound (hr)
1
2
3
4
Holding tank
12
24
Holding time in lab (hr)
00:00
12:00
00:00
Time of assay (h:min)

Fig. 6. Proportion of responsive fish encountered in screening assays using 0.5 g.e./ml of fresh milt as stimulus plotted against individual variables considered as possible influencing factors.


Proportion of responsive fish (%)
1.0
0.5
0.0
07-Mar 27-Mar 16-Apr 06-May 26-May
Date of screening
0% 20% 40% 60%
Proportion of spontaneous fish (%)
0 100 200 300 400
Holding time in compound (hr)
1 2 3 4
Holding tank
0 2 4 7
Holding time in lab (hr)
00:00 12:00 00:00
Time of assay (hr:min)

Methods

Preliminary experiments were carried out with 15 and 20 male fish that had been held in the 2m diameter tanks for five hours and 10 days, respectively, without spawning. Water depth during this time was about 1.5m. The water was drained from the tanks to a depth of 3 cm and maintained at this depth for 30 minutes with continued water flow. The dorsal fins and part of the backs of herring were exposed to air while swimming in this depth of water, and some fish swam sideways on occasion. After the exposure period, I refilled the tank at a rate of about 1.5 cm depth/min and monitored spawning activity.

The role of olfactory cues in initiating spawning under these conditions was investigated in three equivalent trials. The experimental group of each trial consisted of six male fish with both nares occluded with Vetbond tissue adhesive (3M, St. Paul, MN), whereas the control group of six fish had only one naris occluded. For each trial, the control and experimental groups of fish were placed in two equivalent tanks, immediately after olfactory occlusion. The two tanks were adjacent to one another and supplied by the same source of sea water. Both tanks initially contained water at a depth of about 1 meter. After a 30 minute period of acclimatization, the water supply was shut off and the tanks were drained to a depth of about 3 cm. The fish were then left in this depth of water for 15 minutes before their tank was refilled at a rate of about 1.5 cm/min up to the initial depth. The fish were monitored continuously for signs of papilla extension and spawning for a period of 1 hour after the initiation of refilling. This experiment was repeated twice with fresh groups of fish, but the tanks were alternated for the two treatments. Tanks were thoroughly scrubbed and rinsed with sea water between trials. The significance of the response was evaluated with Fisher's exact probability test of pooled results.

Results

All fish in preliminary tests were spawning on the walls of the tank at the first observation time of 1 h after refilling of the tank began. The water in the tank was very cloudy from milt at this time. In the subsequent three tests in which fish had one or both nares occluded, only the tanks with unilaterally-occluded fish showed spawning activity or papilla extension. Approximately 50% (4/6, 3/6, and 2/6) of the fish showed such activity, corresponding to a significant positive response relative to the bilaterally occluded fish (9/18 vs 0/18; P=0.0005). The responding fish started swimming on their sides, perpendicular and close to walls of the tank, approximately 20 minutes after the start of refilling (approximately 35 cm depth of water). Papilla extension was observed in these fish shortly after the start of this swimming behaviour, but not in the remaining fish. By 40 minutes after the start of refilling (approx. 75 cm depth of water), 4/6 and 3/6 fish were spawning on the walls of the tank during the first two trials, respectively, but none spawned in the final trial. No spawning or papilla extension was observed in bilaterally-occluded fish.

5) PROLONGATION OF PHEROMONE RESPONSES IN LARGE TANKS WITH NATURAL SPAWNING SUBSTRATES

I found that the response to the spawning pheromone was over generally within 20 minutes of exposure. However, natural spawning of a school of herring may continue for several hours, apparently including the prolonged spawning of individual fish. The small aquaria I have used for studying spawning of herring are a considerably unnatural environment devoid of physical cues a fish may normally encounter on spawning grounds.

I thus also carried out some experiments with schools of fish in a larger tank containing some natural spawning substrates.

Methods

Pheromone responsiveness in larger tanks was investigated with a group of about 100 fish (including both males and females) in a 3m diameter tank with a 2m x 2m window and benthic natural spawning substrates (rocks covered with the surf grass *Phyllospadix* and the red alga *Rhodomela*). This tank was filled to a 1 m depth with running sea water and was lit from the top by 4 halogen filming lights. Observations were made from a darkened room. Fish were acclimatized to the experimental tank for 5 hours before testing, during which time no spawning or papilla extension was evident. Spawning was induced by injecting three aliquots of 30 ml of testes extract (10 g.e./ml) into the path of the swimming school of fish at intervals of 30 minutes with fine clear plastic tubing from a 60 ml syringe. Two such trials were conducted. During a third trial, several fronds of the floating kelp, *Macrocystis* (also a natural spawning substrate when present) were added to the tank in the area of the schooling fish approximately 30 min after the second addition of testes extract. After a further 30 minutes, a third aliquot of testes extract was then applied. The responses and subsequent spawning activity of the fish were recorded.

Results

Undisturbed fish in the large tanks swam in a school in the upper third of the water column, circling over the deepest portion of the tank, i.e. away from the rocks. About 30s after encountering testes extract, fish ceased to swim in ordered schools and clustered in the area of the pheromone

introduction. Within this cluster, the fish swam close to the water surface in an unordered, circular manner. By 2-3 min after the stimulus, the papilla of most fish was fully extended. Males mainly released milt into the water, but a few fish also spawned on the wall of the tank. Most fish had retracted the papillae and resumed schooling 20 minutes after the first two pheromone applications, even though considerable milt had accumulated in the water. Similar results were obtained in three preliminary experiments, even with additional pheromone applications. The addition of the floating kelp (*Macrocystis*) prior to the third pheromone application changed the swimming pattern of the fish from swimming close to the surface over the deepest part of the tank, away from the rocks, to swimming in mid-water throughout the tank. When the testes extract was subsequently added, schooling was disrupted, papilla extension occurred, and milt was released in the water column as before. However, after this pheromone application the fish continued to spawn for five hours on the surf grass (*Phyllospadix)* and red algae (*Rhodomela*) growing on the rocks, with some also spawning on the *Macrocystis*.

DISCUSSION

The response elicited in herring by spawning pheromones of the milt and testes resembles a "modal action pattern" (MAP) as defined by Barlow (1968, 1977) in that: 1) the response is a sequence of predictable behavioural events triggered by a transient short stimulus, 2) response times are generally greater than the several seconds observed for reflexive sperm release in goldfish (Demski and Dulka, 1984) and 3) the behavioural pattern also occurs in the absence of overt stimulation. The graded nature of the

herring response and its temporal variability (coefficients of variance of response and latency times of 64-67%) appear high for "fixed" action pattern, but are within the range of typical modal action patterns when incorporating variation between individuals (Barlow, 1968, 1977). I have considered the pheromonal response of herring as a modal action pattern to provide a framework for characterizing the behavioural sequence and assessing controlling factors.

Behavioural description

Stacey and Hourston (1982) described the response by herring to a milt stimulus as consisting of 1) disruption of schooling behaviour and rising to the surface of the water ("rising" and "milling"), 2) extension of the gonadal papilla, 3) "substrate testing" by swimming in closer contact with the substrate, and 4) "substrate spawning" with the apposition of the extended gonadal papilla to the spawning substrate and deposition of gametes.

The behavioural sequence elicited in herring by spawning pheromones of the milt and testes in our aquaria and tanks (Fig. 1) is similar to that observed by Stacey and Hourston (1982), except that I did not find substrate testing to be a reliable component of the response to the pheromones in milt and testes. I did observe rapid swimming with the chin and pectoral fins rubbing the aquarium floor, but independently of the addition of spawning pheromone. Such behaviour ceased when I placed the aquarium on a film of diatom growth (results not shown). Thus, in our set-up the behaviour may have been a response to reflections in the aquarium floor. Hay (1985) also questions the contribution of substrate testing behaviour to natural spawning. However, the perpendicular orientation to the tank wall seen in fish during the shallow water trials appears similar to part of the substrate testing behaviour described by Stacey and Hourston (1982).

"Milling" (disruption of the parallel swimming distinctive of schooling behaviour) was described by Stacey and Hourston (1982) and also occurred in our large tank test. The milling behaviour appears to result from a disruption of directed swimming of individual fish as they encounter the spawning pheromone, resulting in a clustering of fish. I also found that the fish rose closer to the surface of the water at this time, in a manner similar to, but not as pronounced as, the rising described by Stacey and Hourston (1982). They also found that both rising and milling occur in response to food stimuli, so are not necessarily specific to the pheromonal response.

I did not measure swimming speeds. However, rapid swimming schools prior to spawning are believed to occur in nature (Hay, 1985), and some experiments introducing aqueous suspensions of macerated testes into net pens resulted in agitation in the fish rather than spawning (H. Kreiberg, unpubl.).

Deduced physiological characteristics:

The three physiological components considered to be part of a modal action pattern are: sensory or central input, a central pattern generator, and motor output effector systems (Shepherd, 1983). These are considered individually below:

Sensory input: I have demonstrated by occlusion experiments that olfaction is the sensory component of the response to the herring spawning pheromones, as it is for the majority of fish pheromones described so far (see Sorensen, 1992). As may be expected for sensory perception (Shepherd, 1983), priming and adaptation to the stimulus were observed, though I was not able to demonstrate refractoriness.

Central pattern generator. The structural correlates of the central pattern generator for the response remain unknown. However, some

physiological characteristics can be inferred from my results. I found that the response is triggered by a transient supra-threshold stimulus and generally stops within 20 minutes. Variability of response and latency time suggests that the output of the central pattern generator is graded, and may be the component that is influenced by factors that have an effect on these times (e.g. stimulus strength, Tables 2, 3).

Effector systems. Three effector systems are likely to be involved in herring spawning. Firstly, relaxation of a gonadal sphincter or the gonoduct may be involved, because I found that anesthesia results in release of sperm and eggs (results not shown). Dulka and Demski (1982) also describe contractions of the sperm duct that are associated with sperm release, but I have not tested for such mechanisms. Secondly, the papilla is extended either due to erectile tissue or extensor muscles. Thirdly, trunk musculature is used for spawning behaviour, because modified swimming movements occur during spawning.

Control of sensitivity and responsiveness to the pheromone.

Our initial hypothesis was that maturity or stress are the factors responsible for changes in responsiveness. I found that sensitivity to the pheromone is variable between individuals, but, other than an indication that females developed pheromonal sensitivity later than the males (Fig. 3A), I found little evidence that sensitivity is related to maturity or sex of the responding fish during the spawning season, though more intensive studies may be needed, and I address this issue in Chapter 4 as well. Also, I was unable to find clear evidence of inhibitory effects of stress on the propensity to spawn or respond to the pheromone in herring. At least a proportion of the fish responded to the pheromone and spawned readily in the assay aquaria: an unnatural environment that could be expected to be stressful. Very high

levels of cortisol in the blood of similar captive herring have been reported (Scott et al., 1991), but I did not find a correlation between high levels of cortisol and pheromone responsiveness in the fish I studied (Chapter 4). However, stress may also be acting in a more indirect manner. For example, sulphated cortisol is a prominent component of milt and urine (Scott et al., 1991), and may act as an olfactory cue to other fish of stressful circumstances and influence responsivity to the spawning pheromone. Exposure to shallow water may thus cause release of such a "stress pheromone" that influences spawning, but this remains to be tested.

My preliminary observations of spawning herring in the commercial fishery (Carolsfeld and Kreiberg, unpublished) suggested that handling stress may also trigger, rather than inhibit, spawning. My shallow-water experiment was designed to test if stress due to crowding and low water level triggered spawning by: 1) stress independent of olfactory cues, or 2) stress-enhanced sensitivity to the spawning pheromone. If spawning is induced directly by stress, I expected no difference in the response of fish that could or could not smell. In contrast, I expected a response only from fish that could smell, if the experiment resulted in stress-enhanced sensitivity to the spawning pheromone. However, I also expected responses to the spawning pheromone to occur within 3-5 minutes of exposure to the pheromone, which was most likely to be present while the water was shallow. However, I found that the response triggered by the shallow water treatment fits neither of these two hypotheses in that it is dependent on olfactory cues but occurs only 20 min after the tank was refilled. No milt was evident in the water at any time, but this does not preclude the release of traces of pheromone in milt or urine. However, these would be more dilute in the full tank than during the shallow water exposure. Thus, the delayed spawning response may be due to a number of other factors which remain to be investigated.

The role of the spawning pheromone in school spawning of herring

The spawning pheromone in herring milt has been postulated to be the principal factor that initiates school spawning of this species (Stacey and Hourston, 1982). However, both the source of the initial milt for triggering mass spawning and requirements for prolonged pheromone-induced spawning remain problematic. Ware and Tanasichuk (1989) have postulated that initial milt in natural spawning of a school of herring is triggered by milt that is squeezed accidentally from some fish, while factors that regulate the prolongation of spawning have not been addressed previously.

My studies found that a proportion of mature fish will commence spawning without overt pheromonal stimulation ("spontaneous" fish). Male fish of this kind are more likely to be the source of initial milt for triggering mass spawning than the accidental squeezing of some fish. I found that "spontaneity" is a character that could be acquired within a period of two hours, can be expressed by both fish that are responsive to the spawning pheromone and those that are not, and is more likely to occur after prolonged holding time in tanks (Table 5). However, I do not yet know what factors lead to spontaneity. Candidate factors include pheromones, possibly other than the spawning pheromone, as these are likely to be of greater concentration in the holding tanks with limited water exchange than in the net pens. In addition, the response to shallow water exposure could be interpreted as induced spontaneity. I have shown that this response is mediated through olfaction and have also suggested that it is due to pheromones.

Prolonged pheromone-induced spawning in a school of herring is likely to include both an enhanced proportion of fish that are responsive to the pheromone and prolonged individual responses. Our characterization of the behavioural response to the spawning pheromone in Pacific herring is one of the first attempts after Tavolga's (1956) early work to investigate the sources

of individual differences in propensity to respond to a pheromone beyond the cruder differentiation of responses in sexually immature and mature fish. Comparisons between Tavolga's findings and ours are illuminating in this context.

The pheromone-induced courtship of the frillfin goby as described by Tavolga (1956) is a similar graded modal action pattern to that of the pheromone-induced spawning of the herring with a comparable time course and individual variability in responsiveness to the pheromone. The propensity to respond to the courtship pheromone in the frillfin goby (considered to be comparable to a behavioural "set" of humans by Tavolga) could be changed by courtship itself, but was considered "spontaneous" by Tavolga (1956) and could not be correlated with evident external characteristics or conditions. I found that the propensity of ripe herring to respond to the spawning pheromone also can change within short periods of time, but in the work of this chapter I did not manage to determine conclusively what factors may contribute to this "set". However, differences in levels of reproductive steroids that are correlated with responsiveness to the pheromone indicate that subtle advances in reproductive maturity may enhance the spawning "set" (Chapter 4). Other undetermined factors no doubt also contribute to the "set", either directly or indirectly, judging by the individual and temporal variability of its expression.

Tavolga (1956) also found that the frillfin goby courted only for approximately 30 minutes in response to the pheromone alone, while courtships of several hours could be induced with additional visual cues of a mate. Pheromone-induced spawning of the herring also appears to rely on additional cues for prolongation of the response. These do not appear to be social visual cues, because even small schools in larger tanks or net pens do not continue to spawn reliably. Two other possible prolongation factors

are the presence and proximity of spawning substrate and exposure to shallow water. Our results suggest that at least the second of these also acts through olfaction. Repeated pheromonal stimuli resulting from the fish swimming through patchy milt over a spawning ground may also play a role in continued spawning. Our results of the individual assays suggested that both priming by low concentrations of pheromone and adaptation to constant levels of the pheromone play a role in the spawning response of herring. Heterogeneous concentrations of milt in the water are thus likely to be the most effective stimulus for induced spawning. Atema (1995) also suggests that the signal-to-background contrast provided by patchiness is essential for responses to odours in the aquatic environment.

Thus, I have found that the spawning pheromone of herring is comparable to other fish releasing pheromones in that it is detected through olfaction and induces a predictable behavioural sequence. I have additionally demonstrated that the response to this pheromone is triggered by a short suprathreshold stimulus and that the resulting modal action pattern is graded based on both individual variability of the responding fish and the nature of the stimulus. The role of the spawning pheromone in the school spawning that is distinctive of the Pacific herring is likely to be modulated by the presence of "spontaneous" individuals to provide initial milt, the proportion of fish responsive to the pheromone, patchiness of milt in the water, and the presence of cues other than the spawning pheromone (which may include the presence of spawning substrate and additional pheromones). Factors contributing to the individual physiological "set" of spontaneous spawning or responsiveness to the spawning pheromone were not fully resolved in this work, but are considered again in Chapter 4.

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CHAPTER 3. REPRODUCTIVE STEROIDS DURING MATURATION IN THE PACIFIC HERRING (*CLUPEA HARENGUS PALLASI*)[2]

ABSTRACT

Concentrations of reproductive steroids were measured in the plasma of captive Pacific herring, *Clupea harengus pallasi*, 1) prior to ovulation and milt production, 2) during the peri-ovulatory and early milt-producing period, 3) during a "ripe" holding period after ovulation and during milt-production, and 4) before and after spawning. 17α,20ß-dihydroxyprogesterone (17,20ß-P), despite being present only in low concentrations in the unconjugated (free) form (< 10 ng/ml), is likely to be the maturation inducing steroid (MIS) in females and was associated with the initiation of milt production in males. Glucuronated 17,20ß-P, free 17α-hydroxyprogesterone (17-P) and free and glucuronated 3α,17α-dihydroxy-5ß-pregnan-20-one (3α,17-P-5ß) were present in high concentrations (140-250 ng/ml) in peri-ovulatory females and newly milt-producing males. This steroid pattern suggests that the low levels of 17,20ß-P are due to glucuronation and competitive conversion of its precursor, 17-P, to free and glucuronated 3α,17-P-5ß. Glucuronated testosterone was the principal steroid in pre-ovulatory and pre-milt producing fish (200-350 ng/ml), coincident with similar levels of glucuronated 11-ketotestosterone in males. After ovulation females did not spawn synchronously until two months later, which may be partially due to

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[2] Portions of this chapter were published in Gen. Comp. Endocrin. **103**: 331-348 with co-authors A.P. Scott (MAFF, Fisheries Laboratory, Lowestoft, Suffolk, England), P.M. Collins (Dept. Evolution, Ecology and Marine Biology, U. Calif. Santa Barabara, USA), and N.M. Sherwood (Dept. Biol., Uvic). Steroid assays were carried out at Lowestoft by J. Carolsfeld with the assistance of Dr. Scott and histology of gonads was carried out by Dr. Collins.

reduced environmental cues in the captive situation, whereas male fish released milt sporadically throughout the ripe holding period. Steroidal indicators of readiness to spawn in females or males were not detected. Rather, levels of all steroids gradually decreased in ripe holding fish (<30 ng/ml) to reach even lower levels (<1 ng/ml) after spawning.

INTRODUCTION

Pacific herring (*Clupea harengus pallasi*) are primitive teleosts whose reproductive physiology is of particular interest because 1) ovulated eggs are held in the ovarian lumen for up to a week in nature (Ware and Tanasichuk, 1989) and several months in captivity (Kreiberg et al., 1982; Hay, 1986) prior to spawning (compared with less than an hour to several days in most fish species (Harvey and Carolsfeld, 1993) and 2) spawning is carried out *en masse* without overt physical interaction between individuals (Schaeffer, 1937). A spawning pheromone present in the milt (Stacey and Hourston, 1982; Sherwood et al., 1991; Carolsfeld et al., 1992) is believed to be important for initiating spawning of this species. However, sexual maturation appears complete, judging by the fully grown gonads and easily expressed gametes, and pheromonal responsiveness is present in both sexes well before spawning (Chapter 1). Thus, it is of interest to study the physiological mechanisms that are involved in the reproductive maturation and preparation for spawning in this species. Levels of reproductive steroids in the blood have been used in a number of more recently evolved fish species as indicators of reproductive development (e.g. Scott et al., 1984, Scott and Canario, 1990), and should be useful in elucidating the mechanisms involved in

reproduction of the herring. Other than our preliminary work (Scott et al., 1991), information is not available on the reproductive physiology of clupeids. In this chapter, I continue our earlier studies by examining the seasonal changes in reproductive steroids found in the blood of captive Pacific herring, with particular focus on the holding period of ripe fish and subsequent spawning.

MATERIALS AND METHODS

Fish:

Adult herring were captured by purse seine in Trincomali Channel, B.C. in mid-February, 1994, prior to ovulation and milt production, and transported by a live holding barge to Departure Bay, Nanaimo, B.C. Fish were maintained as groups of about 1 ton of fish each in 20 x 30 x 5m deep net pens at the Experimental Fish Farm of the Pacific Biological Station (Department of Fisheries and Oceans), without artificial feeding, until after spawning. Also, several hundred fish were transported to the University of Victoria and held at a stocking density of 150 fish per 2000 l tank with refrigerated (12 °C) recirculating sea water and fed frozen zooplankton *ad libitum*.

Sampling:

Fish for sampling of blood and monitoring of maturation were dipped with a 0.5m diameter dip net at random from net pens in Nanaimo after crowding fish to one end of the pen by lifting the bottom of the pen. Fifteen fish were sampled at each time, with approximately equal numbers of each sex. All healthy fish in the pen appeared to have equal

opportunity of being sampled by this process, and were captured approximately 20 minutes after initial disturbance. Sampling was carried out on a weekly basis from late February to late March and every two or three weeks during the remainder of the experimental period until spawning in April. Fish were initially sampled only from pen A until spawning occurred. Fish of the adjacent pen B then were sampled also until spawning two weeks later. Blood samples were collected in November of 1994 from fish captured in February 1994 and maintained in tanks at the University of Victoria.

Blood was sampled from the caudal sinus of the fish within 5 minutes of capture immediately after killing the fish with a quick blow to the head. Heparinized 22G needles and 3 ml syringes were used for sampling. The blood was stored on ice for 0.5 - 2 hr and was then centrifuged at 4 °C for 5 minutes at 2000 x g before the plasma was removed and frozen.

Sex, stage of maturity, body weight, standard length, gonad weight, spermatocrit and milt volume (for spermiating fish) were determined for the fish in each sample. Criteria outlined by Hay (1985) for Pacific herring were used to assign a Hjort maturity index to fish, with the exception, based on Bowers and Holliday (1961), that fish with easily expressible gametes and sticky, transparent eggs were classified only in stage VI (ripe fish) (Table 6). In the present study, none of the females were judged as stage V (mature but not ovulated) or VI+ (partly spent).

Gonadosomatic index (GSI) was calculated as the ratio of the weight of the gonad to the weight of the whole body. Milt volume was determined by measuring the volume of milt that could be easily extruded with abdominal pressure from freshly killed fish. Spermatocrit values were determined in triplicate with milt that was aspirated directly from the gonopore into hematocrit tubes and centrifuged 10 minutes. Spermatocrit

values, obtained only in 1994, were expressed as the proportion of packed sperm in the total sample volume.

The anterior dorsal corner of one ovary of each female fish was fixed in 10% phosphate-buffered formalin or Davidson's fixative (Humason, 1979) for histological examination. This tissue was subsequently dehydrated through a graded series of alcohols and embedded in glycol methacrylate (JB4, Polysciences, Inc.). Sections, 2-3 mm thick, were cut on a Sorvall "PorterBlum" ultramicrotome (MT-1, Sorvall, Inc.) and stained with methylene blue-azure II with a basic fuchsin counterstain (Humphrey and Pittman, 1974).

Analysis of hormones:

Initial screening of reproductive steroids in herring blood was carried out on pools of plasma samples to determine which steroids were of greatest interest to investigate. These pools were made by combining equivalent aliquots of processed plasma (Table 7). Unconjugated ("free") and conjugated forms of 17α, 20α-dihydroxyprogesterone (17,20α-P); 17α,20β-dihydroxy-progesterone (17,20β-P); 17α,20β, 21-trihydroxyprogesterone (17,20β,21-P); 3α,17α-dihydroxy-5ß-pregnan-20-one (3α,17-P-5β); 11-deoxycortisol (11-DOC); testosterone (T); 11-ketotestosterone (11-KT; males only); estradiol (E; females only); 17α-hydroxyprogesterone (17-P; free only) and androstenedione (A; free only) were measured in these samples.

Individual levels of 17-P, 17,20β-P, 3α,7-P-5β, T, and 11KT (free and conjugated forms) and 17-P (free form) were measured in a subset of

Table 6. Sexual maturity stages of herring

Stage	Sexual maturity	Gonad description		
		This thesis	Hjort scale (Bowers & Holliday, 1961)	Hay (1985)
I	Virgin fish; gonad not developed	Not observed	Gonads torpedo- (female) or knife- (male) shaped, 2-3 mm broad	Gonads thread-like, < 2mm broad
II	Start of gonadal development	Not observed	Gonads approx. 1 cm diameter	Gonads ribbon-like, knife- and torpedo-shaped, 3-5 mm broad
III	Gonadal development	As in Hay (1985)	Gonads occupy 1/2 of body cavity	Gonads 5-15 mm broad, Ovaries red-orange, testes reddish grey.
IV	Maturing, gonadal growth (vitellogenesis & spermiogenesis)	As in Hay (1985); late stages only observed, maturing oocyte diameters 650 - 1010 μm	Gonads occupy 2/3 of body cavity, eggs opaque, milt whitish	Gonads extend full length of body cavity with blood vessels visible; ovaries reddish-orange, eggs opaque; testes grey & ooze sperm when sliced
V	Mature; end of vitellogenesis & spermiogenesis: competent for ovulation and milt production	Not observed in females or included as late stage IV; testes white & ooze sperm when sliced, fill whole body cavity; milt not expressible	Gonads occupy whole body cavity, some transparent oocytes, milt white but not running; blood vessels prominent in ovaries.	Gonads bulging & occupy whole body cavity, gametes extruded with pressure; oocytes transparent gold-yellow & adhesive; testes white; no blood vessels visible.
VI	Ripe: ovulated & producing milt	As in stage V of Hay (1985); all eggs ovulated, transparent, adhesive, diameter about 1150 μm; eggs and milt flow easily with or without pressure	Roe and milt running	As in stage V, except gametes flow easily without pressure
VI+	Partly spent	Males: testes reduced in size, slightly bloodshot, thinning at edges	Not described	Not described
VII	Spent	As in Hay (1985); blood extruded from gonopore with pressure in recently spent fish	Ovaries slack with residual eggs, testes baggy, bloodshot.	Gonads slack, ovaries with some residual eggs, testes limp & bloodshot.
VIII	Recovering	As in Hay (1985)	Not described	Gonads small & wine-coloured; blood vessels prominent.

the available plasma samples from 1994 representing stages IV-VII and the full range of sampling dates (Table 7). Samples to be used were selected from each sampling date in the order of capture to ensure randomness. Plasma pools of stage III fish (maintained in captivity after spawning until gonadal recrudescence and sampled on Nov. 2, 1994) were included in the assays of individual plasmas. To avoid interassay variability, all samples of each sex were assayed together for each steroid form.

Herring plasma was found to contain large amounts of non-polar lipids that appeared to interfere with steroid extraction, so samples were semipurified on C_{18} Sep-Pak cartridges prior to steroid analysis. 100 µl of plasma was diluted to 0.5 ml with distilled water and then loaded onto a primed cartridge. The cartridge was then rinsed with a further 2 ml of water, and the steroids eluted with 4 ml of methanol. The methanol was removed under a stream of nitrogen at 45°C and the resulting residue was reconstituted with 100 ml of distilled water. Effectiveness of the semi-purification was evaluated by spiking 4 plasma samples from November, 1994 (stage II fish known to have low levels of reproductive steroids) with 250 ng/ml (final concentration) each of unconjugated 17,20β-P, T and 11-KT and glucuronated 17,20β-P and T. These samples were purified, extracted and analysed in a fashion comparable to the other plasma samples.

Free and conjugated steroids were extracted from the semipurified plasma as described by Scott and co-workers (Scott and Vermeirssen, 1994; Scott and Sorensen, 1994). Briefly, free steroids were extracted directly from the 100 ml of reconstituted Sep-Pak-purified plasma with 4 ml of ether. The aqueous layer was then dried in a vacuum centrifuge, dissolved in 20ml of distilled water, and solvolysed with trifluoroacetic acid

Table 7. Morphological characteristics of fish sampled for blood[1]

Maturity Description	Stage[2]	Sampling date	Source Pen	Weight (g)	Length (cm)	GSI[3] %	Spcrit[4] (%)	Milt vol. (ml)	n	Plasma sample analysed as: pool	individual
Females											
Pre-vitellogenic	III	Nov. 2	UVic	177 ± 14 [a]	221 ± 6	6.0 ± 1.6 [a]			4	x	
Vitellogenic	IV	Feb. 21	A	93 ± 7 [b,c]	200 ± 4	22.1 ± 0.8 [b]			7	x	x
	IV	Mar. 2	A	103 ± 7 [b,c]	211 ± 7	19.3 ± 1.1 [b]			8		x
Ripe (ovulated)	VI	Mar. 7	A	104 ± 8 [b,c]	204 ± 4	26.6 ± 0.7 [b]			5		x
	VI	Mar. 31	A	105 ± 12 [b,c]	202 ± 9	24.4 ± 1.9 [b]			5		x
	VI	Apr. 22	B	117 ± 10 [b]	207 ± 5	25.2 ± 1.2 [b]			7		x
Spent	VII	Apr. 30	B	82 ± 4 [b,c]	200 ± 3	1.5 ± 0.1 [a]			10		x
Recovering	VIII	Jun. 2	A	63 ± 8 [c]	196 ± 11	0.01 ± 0.01 [a]			4	x	
Males											
Spermatogenetic	III	Nov. 2	UVic	120 ± 13 [a]	166 ± 30	9.0 ± 0.9 [a]			6	x	
Spermiogenic	IV	Feb. 21	A	91 ± 9 [a,b]	206 ± 5	17.4 ± 2.4 [b,c]			4	x	x
	IV	Mar. 2	A	87 ± 12 [a,b]	198 ± 8	14.6 ± 2.2 [b]			4		x
Mature	V	Feb. 21; Mar. 2	A	95 ± 14 [a,b]	201 ± 11	17.8 ± 1.1 [b,c]			4		x
Ripe	VI	Mar. 2 & 7	A	107 ± 4 [a]	206 ± 2	20.4 ± 1.0 [b,c]	99.1 ± 0.3 [a]	1.3 ± 0.3 [a]	12		x
	VI	Mar. 31	A	102 ± 7 [a,b]	205 ± 4	21.4 ± 1.2 [c]	89.8 ± 2.1 [b]	0.9 ± 0.2 [a]	10		x
	VI	Apr. 22	B	114 ± 13 [a]	214 ± 6	22.9 ± 1.1 [c]	83.8 ± 1.5 [b]	1.5 ± 0.4 [a]	5		x
Partly spent	VI+	Apr. 21,22, 30	A & B	81 ± 11 [a,b]	198 ± 7	15.2 ± 1.9 [b]	82.0 ± 1.1 [b]	0.5 ± 0.0 [a]	5		x
Spent	VII	Apr. 21 & 30	A & B	78 ± 6 [b]	197 ± 5	1.3 ± 0.1 [a]			12		x
Recovering	VIII	Jun. 2	A	70 ± 7 [b]	199 ± 6	0.01 ± 0.00 [a]			10	x	

[1] Means ± S.E.; values with different superscript letters are significantly different (α=0.05)

[2] See Table 1 for description of reproductive stages

[3] Gonadosomatic index = weight of gonad/total weight of fish

[4] Spermatocrit = proportionate volume of seminal fluid in milt (%)

(TFA)/ethyl acetate (1.4:100) overnight at 45°C to free sulphated steroids. The freed steroids were extracted with ether after removing the TFA/ethyl acetate under a stream of nitrogen. Finally, glucuronated steroids were freed from the aqueous residue of the solvolysed plasma by digestion overnight with *Helix* aryl sulfatase /glucuronidase (Sigma) at 37°C in an acetate buffer (pH 5.3). The freed steroids from this digestion were also extracted with ether. All ether extracts were dried down in a 45°C water bath and reconstituted in 1 ml of assay buffer (0.1 M phosphate buffer with 0.1% bovine serum albumin and 0.1% sodium azide) and stored frozen in this buffer until analysis. The radioimmunoassays I used have been described and characterized elsewhere (Scott et al., 1984; Canario and Scott, 1990b).

Concentrations of conjugated steroids are reported in ng free steroid equivalents/ml, as measured in the assays (i.e. the equivalent quantity of the steroid as a free form), to facilitate comparisons of free and conjugated steroid levels.

Identification of immunoreactive steroids:

The steroids in herring blood that react in the radioimmunoassays for 17-P, 17,20β-P, and 3α,17-P-5β were identified by comparing the elution of the immunoreactive compounds with that of authentic standards by high-pressure liquid chromatography (HPLC) and, for the latter two steroids, by thin layer chromatography (TLC).

100 µl samples for HPLC were taken from the free and glucuronated fractions of a pool of plasma from five ripe male fish. The samples were each spiked with 2000 counts/ml of tritiated 17,20β-P and 17-P (free fraction) or 17,20β-P, 17-P, and 3α,17-P-5β (glucuronide fraction). Chromatography was carried out on a Varian Dynamax C_{18} analytical

column with a Gilson HPLC. A gradient of 28.6% B to 100%B in 50 min was used for elution after 10 minutes at initial conditions of 28.6% B (Solvent A = 0.01% TFA; solvent B = 0.01%TFA in 70% acetonitrile). A flow rate of 0.5 ml/min was used, and 0.25 ml fractions were collected. Radioactivity in 150 µl of each HPLC fraction was measured directly. The remaining two 50 µl aliquots of each fraction were used for radioimmunoassays. 17-P and 3α,17-P-5β were assayed in the fractions from the free steroid pool, whereas 17,20β-P, and 3α,17-P-5β were assayed in the fractions of the glucuronated steroid pool. The identity of other compounds cross-reacting in the 3α,17-P-5ß assay was proposed by comparison with elution positions of other authentic 3α,5β-reduced steroids (Scott, unpublished data).

Thin layer chromatography was carried out with a pooled sample of the glucuronide fraction of plasma from five ripe male fish. This sample (representing 37.5 ml of plasma) was extracted with 1 ml of diethyl ether, 75 µl of which was applied to one lane of a silica gel TLC plate (type LK6DF, Whatman), together with 2000 counts of ^{3}H 17,20β-P as internal standard. The plate was developed with chloroform/ethanol (50:2) for 40 minutes. Strips of the plate, 0.5 cm in width, were scraped off and each extracted with 1 ml of assay buffer. These fractions were assayed for 17,20β-P and 3α,17-P-5β immunoreactivity and the elution pattern was compared with expected elution positions of authentic standards.

Data presentation and statistical analysis:

Morphological characters, hormone levels and conjugation ratios were compared among reproductive stages and sampling dates with single factor ANOVAs followed by multiple comparisons of means with Tukey's multiple t-tests (Zar, 1974), using a significance level of 0.05.

Gonadosomatic indices and spermatocrit values were arcsine transformed for this analysis, whereas hormone levels and conjugation ratios were log transformed to ensure normality. Multiple correlation analysis was also carried out between transformed gonadosomatic indices, spermatocrit values, and hormone levels, as well as between hormone levels of fish grouped by gender and "maturing" or "ripe" (Microsoft Excel 5.0, 1994).

All values are expressed as the mean ± standard error of the mean in the text, figures and tables unless indicated otherwise.

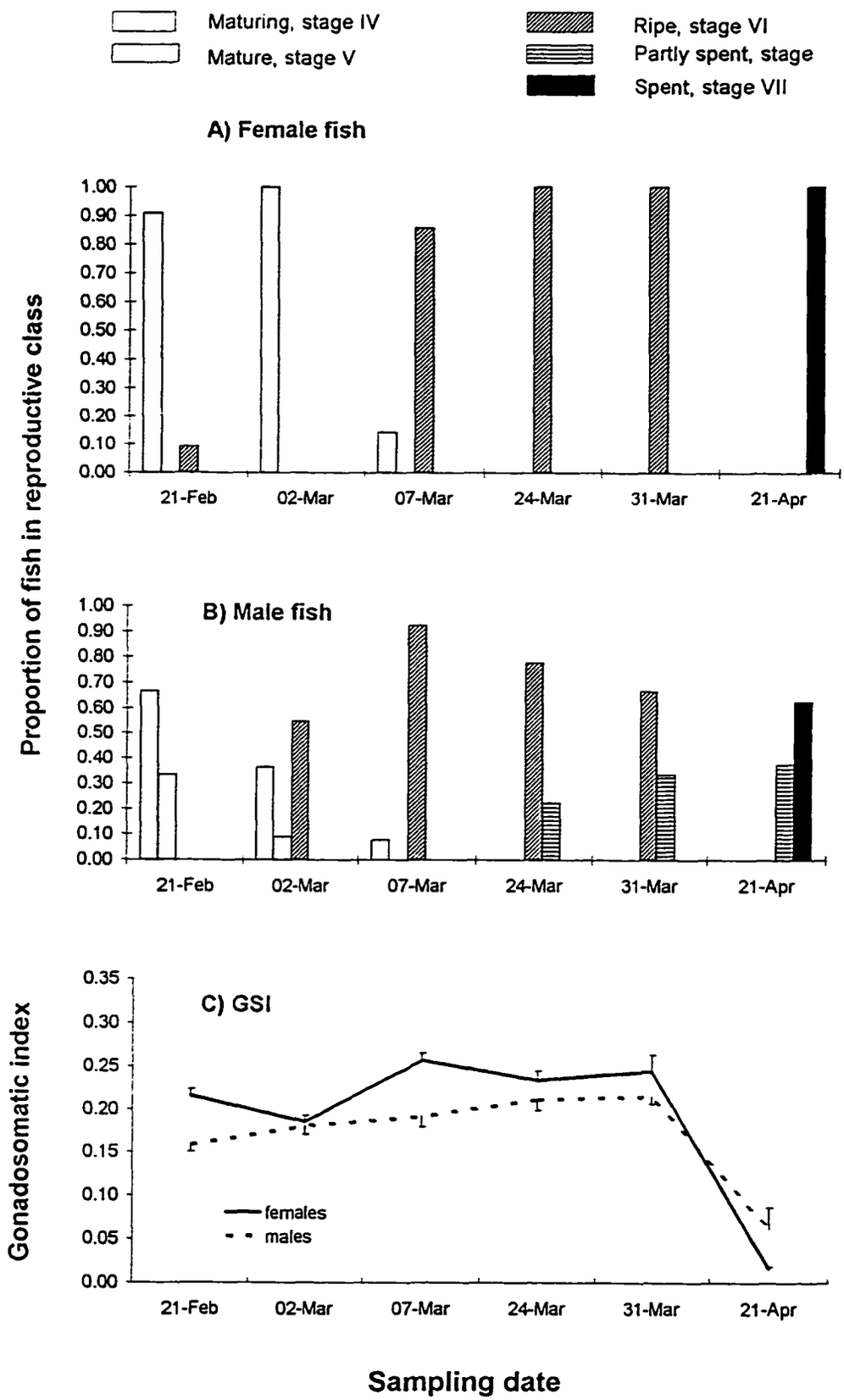
RESULTS

Pattern of sexual maturation in net pens:

A small proportion of female fish may have been ovulated at the time of capture, whereas the remaining female fish in the net pens matured later and more synchronously than did the male fish in our study (Fig. 7). Milt release appeared to have occurred in some fish throughout the ripe holding period (although I have no evidence that spawning behaviour occurred) and not all males were fully spent even after females of both pens had spawned fully. Spawning ofeach pen occurred within a single day: pen A on April 20th and pen B on April 29th. In addition, a wild school of herring spawned in the vicinity of the captive herring on March 7th, shortly after the majority of females had ovulated. Some milt washed through the pens from this spawning but did not elicit spawning.

No stage V (mature) females and few stage V males were encountered in our study.

Fig. 7. Seasonal changes in sexual maturity of captive herring of netpen A during the 1994 reproductive season. A) and B): distribution of reproductive classes within each sex from a total sample of 15 fish (see Table 6 for description of reproductive classes). C) gonadosomatic index of male and female fish from the same samples.


Maturing, stage IV
Mature, stage V
Ripe, stage VI
Partly spent, stage
Spent, stage VII
A) Female fish
B) Male fish
C) GSI
Proportion of fish in reproductive class
Gonadosomatic index
females
males
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.35
0.25
0.15
0.05
21-Feb
02-Mar
07-Mar
24-Mar
31-Mar
21-Apr
Sampling date

Morphological characteristics:

Fish in the subset from which blood samples were analyzed, other than the larger female fish sampled in November and smaller spent male fish, did not differ significantly in size between reproductive classes (Table 7). The gonadosomatic index (GSI) increased from stage III to stage VI (postovulation and spermiating) and remained high throughout the subsequent "ripe" holding period in both sexes. At the end of the holding period, the GSI dropped rapidly in females and more gradually in males during spawning (Fig. 7).

Large oocytes (650-1010 mm diameter) with uniformly granular yolk-filled cytoplasm, a cell wall of externus, internus, and subinternus layers, and a follicular envelope of concentric layers of granulosa and thecal cells were predominant in stage IV ovaries (Fig. 8a,b). Previtellogenic and atretic oocytes were also present. Stage VI ovaries contained large ovulated eggs (up to 1150 mm diameter) with large yolk globules in the cytoplasm (Fig. 8c). The eggs possessed a membrane composed of externus, internus and subinternus layers, but no follicular layer (Fig. 8d). Previtellogenic oocytes and some degenerating follicles were present adjacent to the ovarian wall in stage VI ovaries, but were absent in the lumen. No histological difference was found between recently ovulated ovaries and those of fish that had held ovulated eggs for over two months. Spent (stage VII) ovaries were characterised by an empty ovarian cavity delineated by pronounced folds in the ovarian epithelium. Only previtellogenic oocytes and degenerating unspawned eggs were observed in stage VII ovaries. The proportion of seminal plasma in the milt of ripe holding males increased from about 1 to 18% after the initiation of spawning, as indicated by a significant decrease of average spermatocrit values (Table 7). Average expressible milt volume, on the

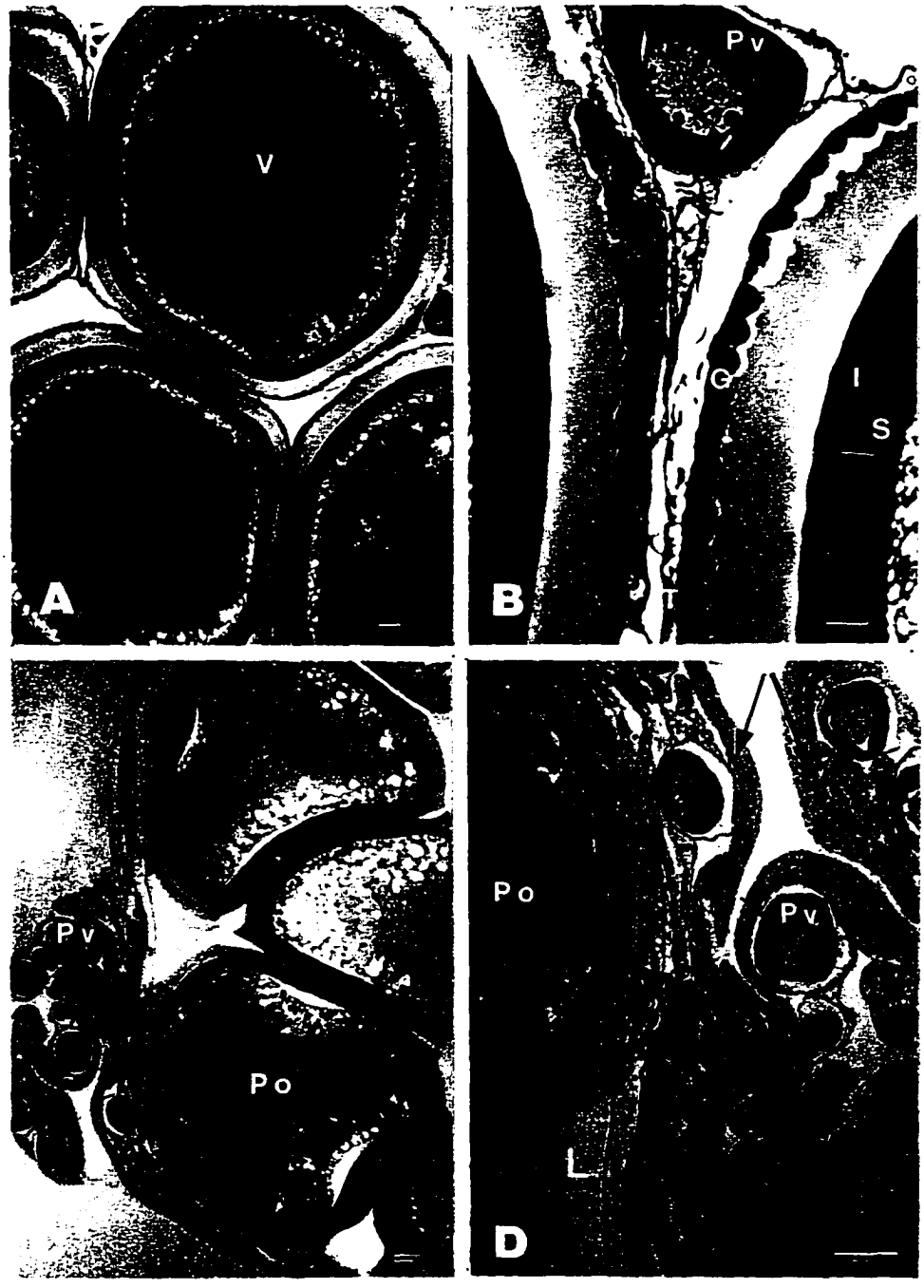
Fig. 8. Light micrographs of herring ovaries before (A and B) and after (C and D) ovulation.

A) Stage IV ovary showing portions of four pre-ovulatory follicles containing large vitellogenin-filled oocytes (V). Scale bar = 100μm.

B) Detail of pre-ovulatory follicles of stage IV ovaries showing concentric layers of thecal (T) and granulosa (G) cells surrounding the oocytes and the externus (E), internus (I), and sub-internus (S) of the oocyte membrane. Pre-vitellogenic oocytes (Pv) are present amongst the follicles. Scale bar = 25 μm.

C) Stage VI ovary with post-ovulatory eggs (Po) in the lumen (L). Granules of vitellogenin in pre-ovulatory oocytes have coalesced into yolk droplets. Pre-vitellogenic oocytes (Pv) are present only in the ovarian cortex. Scale bar = 100 μm.

D) A portion of a postovulatory egg (Po) in the ovarian lumen (L) adjacent to the ovarian cortex. Only the egg membrane layers are present around the egg, whereas the thecal and granulosa layers are absent. Remnants of follicles (arrows) and pre-vitellogenic oocytes (Pv) are present only in the ovarian cortex. Scale bar = 100 μm.


V
A
Pv
G
I
S
B
Pv
Po
Po
Pv
L
D

other hand, did not show a distinct temporal trend (Table 7). Histological examination of milt-producing fish indicated that the fish had completed spermiation, evidenced by the lack of spermatid-containing Sertoli cysts in the testicular wall.

Chromatographic identification of immunoreactive steroids:

The 17-P immunoreactivity (ir) in the free steroid fraction of plasma samples (Fig. 9A) and 17,20β-P ir in the glucuronide fractions (Fig. 9B) coeluted with authentic standards from the HPLC, indicating that these are, in fact, the only steroids measured in these assays. No 17-P was detected in the glucuronide fraction, but a small peak coincident with the 3α,17-P-5β elution position suggests a slight cross-reactivity with this steroid.

The 3α,17-P-5β ir eluted primarily at a position most closely coinciding with the normal elution of 3α,17-P-5β (Scott, unpublished), but small amounts also coeluted with 3α,17,20β-triol-5β-pregnan (3α,17,20β-P-5β) and in other unidentified peaks. These identified compounds react in a parallel fashion to 3α,17-P-5β in the RIA used (Scott, unpubl. obs.). The 3α,17-P-5β peak area represented about 75% of total 3α,17-P-5β ir.

On TLC (Fig. 10), the principal peak of 17,20β-P ir eluted coincidentally with authentic 17,20β-P. 3α,17-P-5β ir eluted primarily at the expected position of authentic 3α,17-P-5β (78%), but 3α,17,20β-P-5β (9.5%) and other unidentified peaks (12%) were also found (Fig. 10). The identity of 3α,17,20β-P-5β was confirmed by another assay that cross-reacts with this steroid (Scott et al., 1996).

Fig. 9. Identification of 17-hydroxyprogesterone (17-P); 17α,20β-dihydroxyprogesterone (17,20β-P) and 3α,17α-dihydroxy-5β-pregnan-20-one (3α,17-P-5β) immunoreactivity (ir) in free (A) and glucuronated (B) steroid fractions of pooled plasma of ripe male herring by high pressure liquid chromatography (HPLC). See text for methodological details. Abbreviation of 3α,17,20ß-P-5ß refers to 3α,17α,20ß-triol-5ß-pregnan.

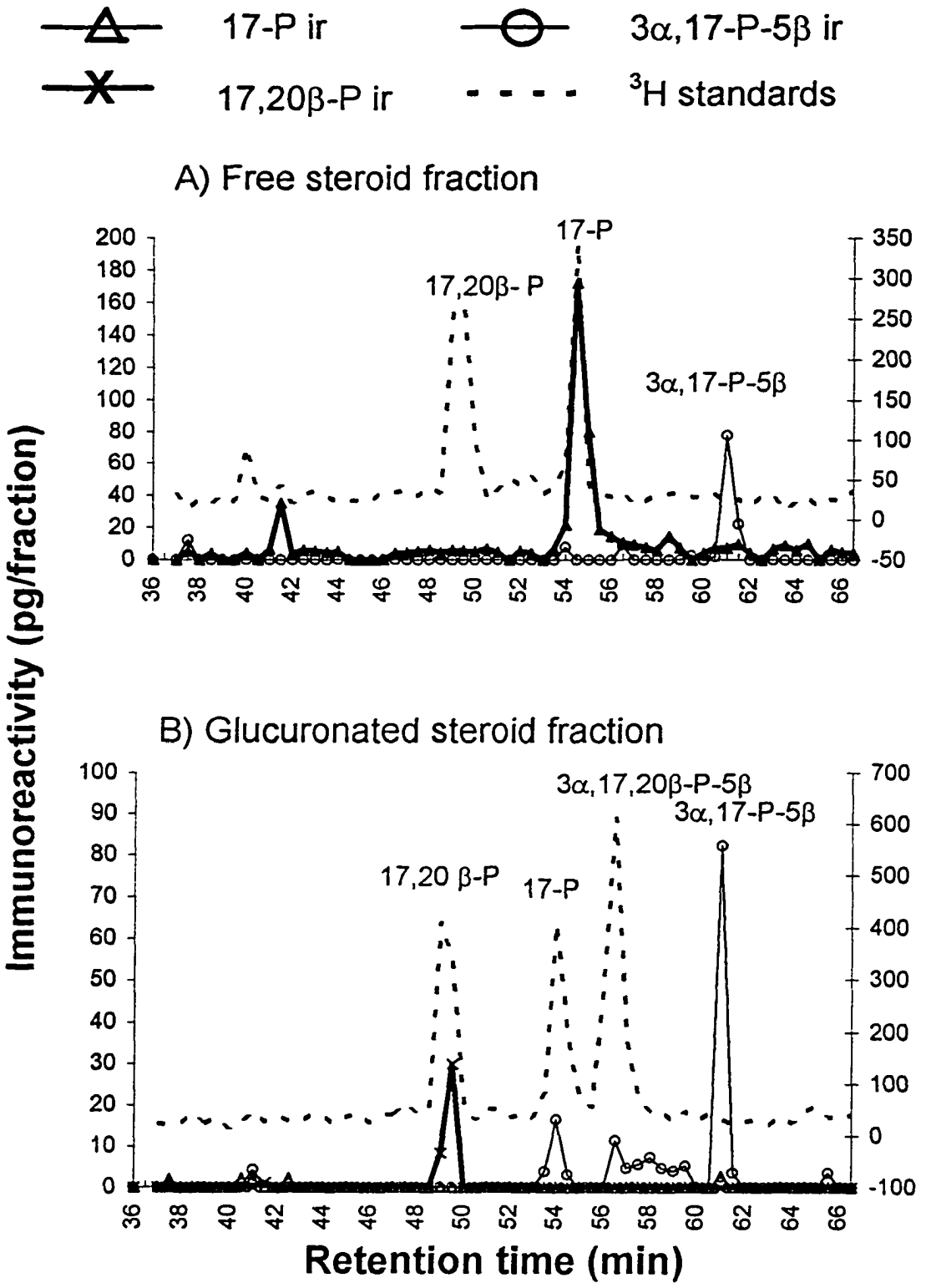
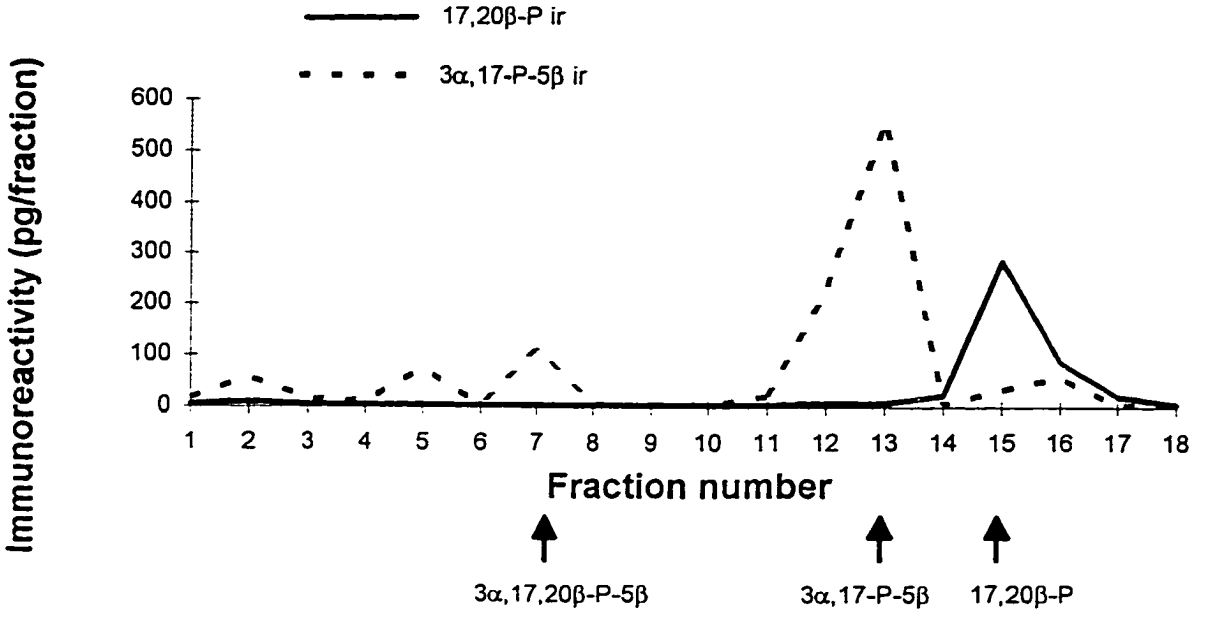

17-P ir
3α,17-P-5β ir
17,20β-P ir
3H standards
A) Free steroid fraction
17-P
17,20β- P
3α,17-P-5β
B) Glucuronated steroid fraction
3α,17,20β-P-5β
3α,17-P-5β
17,20 β-P
17-P
Immunoreactivity (pg/fraction)
Retention time (min)

Fig. 10. Identification of 17,20β-P and 3α,17-P-5β immunoreactivity (ir) in the glucuronated steroid fraction of pooled plasma of ripe male herring by thin layer chromatography (TLC). Fractions represent eluates of sequential 0.5 cm scrapings from the TLC lane. Arrows indicate expected elution position of standards. Abbreviations of steroid names as in Fig. 10.


17,20β-P ir
3α,17-P-5β ir
Immunoreactivity (pg/fraction)
600
500
400
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction number
3α,17,20β-P-5β
3α,17-P-5β
17,20β-P

Steroid recoveries:

Mean recovery of free steroids from purified and extracted plasma that had been spiked with synthetic steroids was 77% (17,20ß-P : 77 ± 11%, T: 72 ± 3%, KT: 81 ± 3%), whereas recovery of glucuronated 17,20ß-P was 101 ± 4% and that of glucuronated T was 73 ± 2%. No correction for recovery rates was made in the data.

Seasonal variation of steroids in pooled plasma samples:

17-P, 17,20β-P, 3α,17-P-5β, T, and 11-KT showed distinguishable changes in concentration in relation to sexual maturity in pooled plasma samples and were studied further in individual plasmas. No major changes in levels of 17,20α-P, 17,20β,21-P and estradiol were found in the samples used, whereas minor changes in free androstenedione in male fish and free 11-deoxycortisol in female fish were detected, but not investigated further.

Seasonal variation of steroids in individual plasmas:

Free steroids, females: 17-P was the major form in the free steroid profiles of plasmas, with a marked peak at ovulation (Fig. 11). Levels were low before ovulation (stages III and IV), and became progressively reduced after ovulation, though still present until spawning. The steroid was not detectable or very low after spawning. Free 3α,17-P-5β levels mirrored those of 17-P, whereas free T and 17,20β-P, both low throughout, had small peaks in stage IV fish (late vitellogenic and preovulatory fish, respectively).

Fig. 11. Seasonal changes in plasma steroids of female herring. Abbreviations of steroid names are as in Fig. 10, with the addition of testosterone (T). Description of reproductive maturity stages are given in Table 6. Asterisks (*) indicate values significantly higher than unmarked values whereas δ indicates values significantly lower than unmarked values (Tukey's multiple range test, $\alpha = 0.05$).

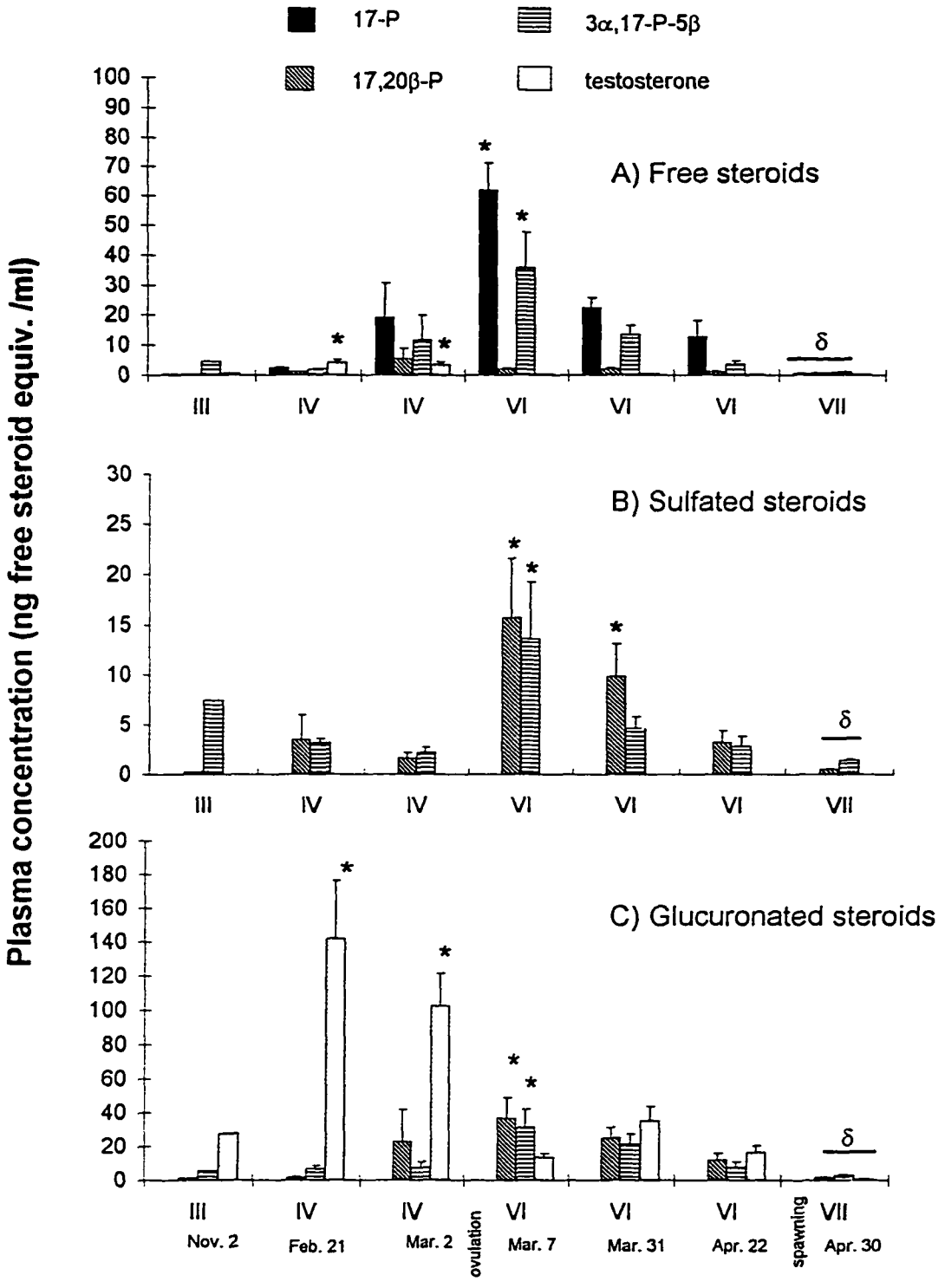
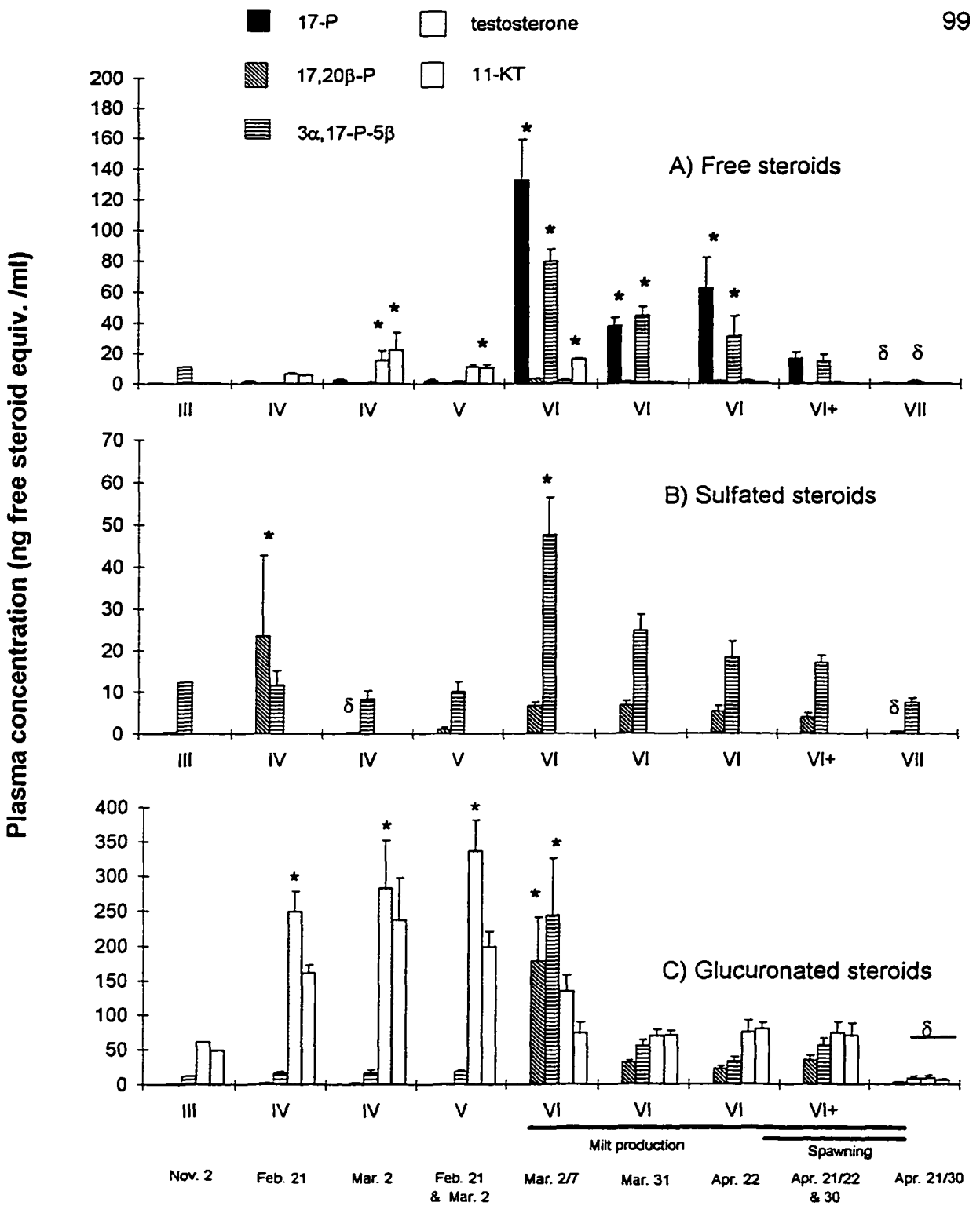

17-P
3α,17-P-5β
17,20β-P
testosterone
A) Free steroids
B) Sulfated steroids
C) Glucuronated steroids
Plasma concentration (ng free steroid equiv. /ml)
III
IV
IV
VI
VI
VI
VII
Nov. 2
Feb. 21
Mar. 2
ovulation
Mar. 7
Mar. 31
Apr. 22
spawning
Apr. 30
Sexual maturity (Hjort index)/Sampling date

Fig. 12. Seasonal changes in plasma steroids of male herring. Abbreviations of steroid names as in Fig. 9 with the addition of testosterone (T) and 11-ketotestosterone (11-KT). Reproductive maturity stages are described in Table 6 and annotation of significant differences is described in Fig. 12.


17-P
testosterone
17,20β-P
11-KT
3α,17-P-5β
A) Free steroids
B) Sulfated steroids
C) Glucuronated steroids
Plasma concentration (ng free steroid equiv. /ml)
III
IV
IV
V
VI
VI
VI
VI+
VII
Milt production
Spawning
Nov. 2
Feb. 21
Mar. 2
Feb. 21 & Mar. 2
Mar. 2/7
Mar. 31
Apr. 22
Apr. 21/22 & 30
Apr. 21/30
Sexual maturity (Hjort index) & Sampling date

Free steroids, males: T and 11-KT were the most abundant free steroids in the blood of stage IV and V males, but were reduced in ripe (stage VI) fish during most of the milt-producing period (Fig. 12). Free 17,20ß-P was relatively low throughout, but showed a significant small peak in milt-producing fish in early March. 17-P and 3α,17-P-5β were the most abundant steroids in stage VI (ripe) fish that had recently initiated milt production, but were reduced in ripe fish later in the season. All of the steroids measured were low in stage VII (spent) and stage III (early gonadal growth) fish.

Sulphated steroids: In females, both sulphated 17,20β-P and 3α,17-P-5β were relatively high shortly after the majority of the fish in the pond had ovulated, but were low at other times (Fig. 11). The slightly elevated levels of sulphated 3α,17-P-5β in the plasma pool of stage III fish is of unknown significance. In males, sulphated 17,20β-P was high in two out of four late stage IV fish, and was slightly elevated in recently ripe fish, but was generally low throughout (Fig. 12). Levels of sulphated 3α,17-P-5β generally reflected levels of free 3α,17-P-5β in these fish.

Glucuronides: Glucuronated steroids were the most abundant steroid form in the blood plasma of both male and female fish (Figs. 11, 12). T glucuronide was high just before ovulation or the start of milt production, coincidental with high levels of glucuronated 11-KT in males. Levels of glucuronated androgens were low at other times in both sexes. Concentrations of the glucuronated progesterones (17,20β-P and 3α,17-P-5β) were high at ovulation and the start of milt production, but were reduced in ripe holding fish and were low otherwise. Glucuronation ratios (concentrations of glucuronated form/free form) of all steroids, other than 3α,17-P-5ß, were highest during the ripe holding period of both sexes

Fig. 13. Seasonal changes in glucuronation ratios of 3α,17-P-5 (A) and other steroids (B) in female herring plasma. Glucuronation ratios were calculated as the ratio of the concentration of a conjugated steroid to the concentration of the related free steroid in individual fish (both concentrations were expressed as free steroid equivalents). Abbreviations of steroid names and indications of significant differences as in Figs. 11 and 12.

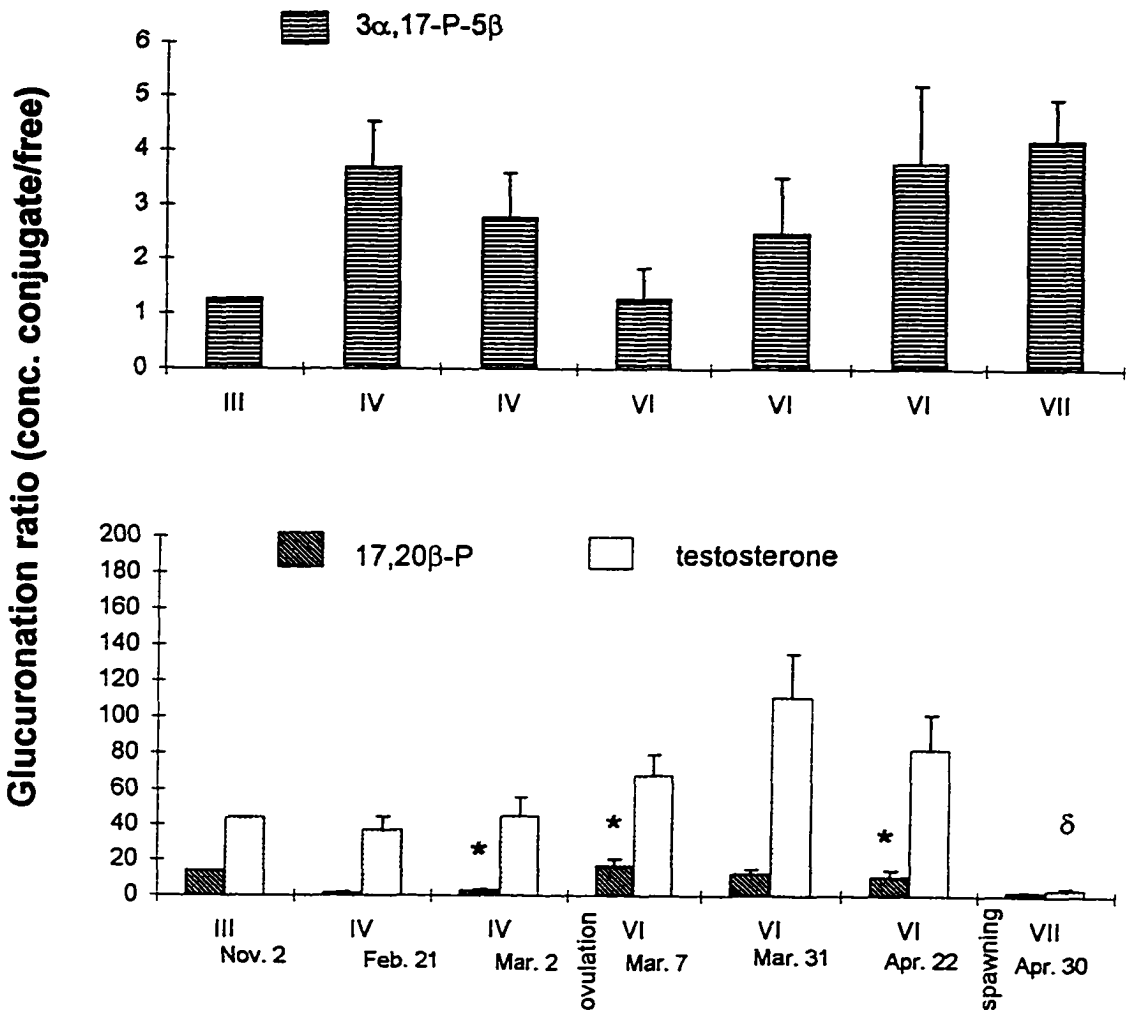
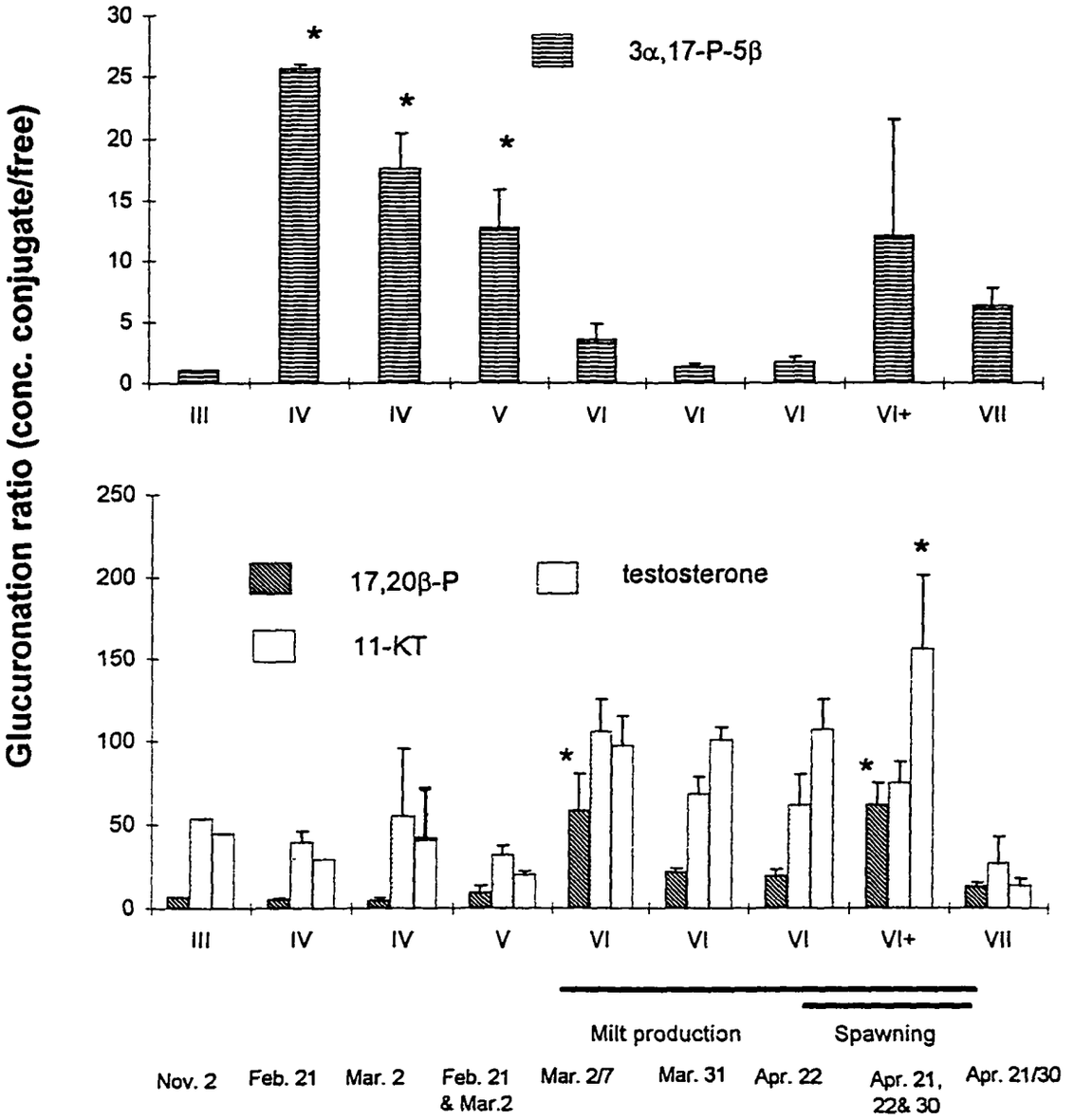

3α,17-P-5β
6
5
4
3
2
1
0
III
IV
IV
VI
VI
VI
VII
17,20β-P
testosterone
200
180
160
140
120
100
80
60
40
20
0
*
*
*
δ
III
Nov. 2
IV
Feb. 21
IV
Mar. 2
ovulation
VI
Mar. 7
VI
Mar. 31
VI
Apr. 22
spawning
VII
Apr. 30
Glucuronation ratio (conc. conjugate/free)
Stage of sexual maturity & sampling date

Fig. 14. Seasonal changes in glucuronation ratios of 3α,17-P-5 and other steroids in male herring plasma. Definition of glucuronation ratios and abbreviations of steroid names as in Fig. 13.



Stage of sexual maturity & sampling date

(Fig. 13). The glucuronation ratio of 3α,17-P-5ß, although low and without a significant trend in females, was highest in males prior to milt production, reduced during ripe holding, and slightly elevated (but highly variable) during spawning (Fig. 14).

Correlation between hormone levels and morphological indicators of maturity:

Significant correlations between morphological indicators of maturity and levels of steroids in the blood were low and present for only a few steroid forms (Table 8). Levels of sulphated 3α,17-P-5β were positively correlated with the gonadosomatic index in maturing female fish, whereas levels of glucuronated T were negatively correlated with gonadosomatic index in ripe females. No other significant correlations were found between levels of the steroids measured and gonadosomatic index of female fish.

Levels of glucuronated T were positively correlated with gonadosomatic index of maturing male fish, whereas no steroid levels were signficantly correlated with gonadosomatic index of ripe males. However, levels of free 17-P and 17,20β-P, all forms of 3α,17-P-5β, and glucuronated T were negatively correlated with spermatocrit, and levels of free 17-P and 17,20β-P were also positively correlated with expressible milt volume.

Correlations between steroid levels of individual fish:

Female fish: Levels of free 17,20β-P and 3α,17-P-5β were significantly correlated with levels of 17-P in both maturing and ripe females, whereas those of free T were not in maturing females and were too low in ripe fish for comparison. Levels of the glucuronated form of all

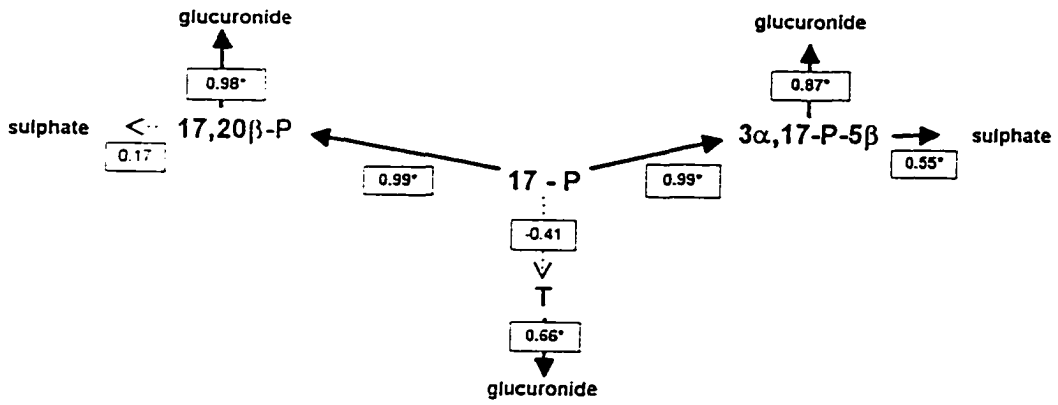
Table 8. Correlation between plasma steroid levels and morphological indicators of sexual maturity in stage IV and ripe herring

		Female		**Male**			
		GSI		GSI			
Steroid	*form*	*stage IV*	*ripe*	*stage IV*	*ripe*	*milt vol.*	*spermatocrit*
17P	free	0.36	0.11	0.23	-0.18	0.42*	-0.44*
17,20β-P	free	0.42	-0.17	0.24	-0.11	0.38*	-0.62*
	sulf	0.36	0.13	-	-0.16	-0.06	-0.18
	gluc	0.37	-0.08	-0.07	-0.20	-0.09	-0.40
3α,17-P-5β	free	0.39	0.14	0.49	-0.11	0.20	-0.61*
	sulf	0.55*	0.18	0.02	-0.26	-0.10	-0.46*
	gluc	0.43	-0.07	0.47	-0.19	-0.11	-0.41*
T	free	-0.09	-	0.14	-0.11	-0.19	-0.17
	gluc	0.24	-0.53*	0.62*	-0.08	-0.10	-0.42*
11-KT	free	-	-	0.19	-0.09	-0.19	-0.22
	gluc	-	-	0.34	0.04	-0.15	-0.06
milt vol.		-	-	-	0.40*	-	-0.19
spermatocrit		-	-	-	-0.13	-0.19	-
sample size		15	17	12	28	28	28

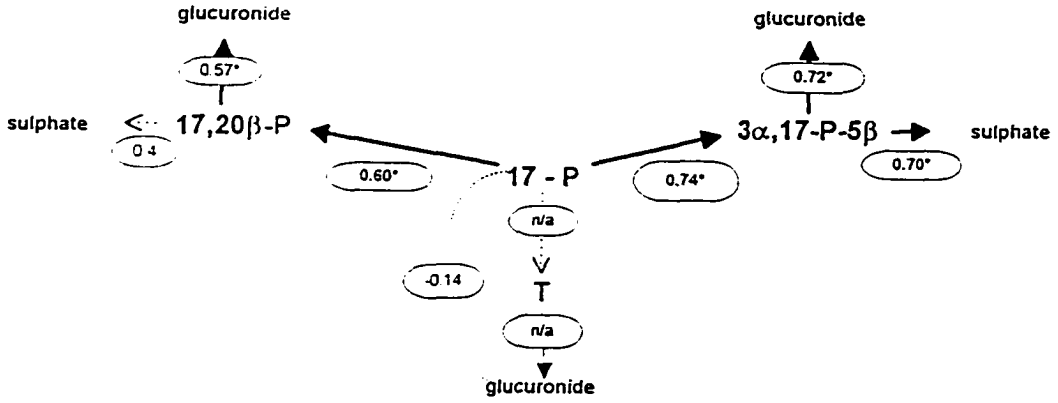
* significant bivariate correlation coefficient (α = 0.05)

Fig. 15. Correlation between plasma levels of metabolically related steroids in A) maturing female fish (pre-ovulated stage IV; n = 15) and B) ripe female fish (ovulated, stage VI; n = 17). Arrows indicate metabolic pathways, whereas numbers in boxes indicate bivariate correlation coefficients between levels of the respective steroid forms. Significant correlations (α = 0.05) are indicated by an asterisk (*) and a solid line of the arrow. Abbreviations of steroid forms as in Fig. 13.

A) Maturing female fish (stage IV)



B) Ripe female fish (stage VI)



steroids were significantly correlated with those of their respective free forms (where measurable), whereas the levels of the sulphated form were significantly correlated with levels of free 3α,17-P-5β but not 17,20β-P in both maturing and ripe fish. Correlations among the progesterones were very high in maturing fish, but of moderate magnitude in ripe fish (Fig. 15).

Male fish: Levels of 17-P were significantly correlated only with those of 17,20β-P in maturing male fish, whereas they were signficantly correlated with levels of both 17,20β-P and 3α,17-P-5β in ripe male fish. Levels of free 11-KT were highly correlated with levels of free T in both maturing and ripe male fish, but levels of T were not correlated with levels of 17-P in either group of fish. Of the conjugated steroids measured, only levels of 11-KT glucuronide were significantly correlated with levels of the free steroid in maturing male fish, and, in ripe fish, only levels of glucuronated T and 11-KT and of sulphated 3α,17-P-5β were significantly correlated with levels of the free steroid forms (Fig. 16).

DISCUSSION

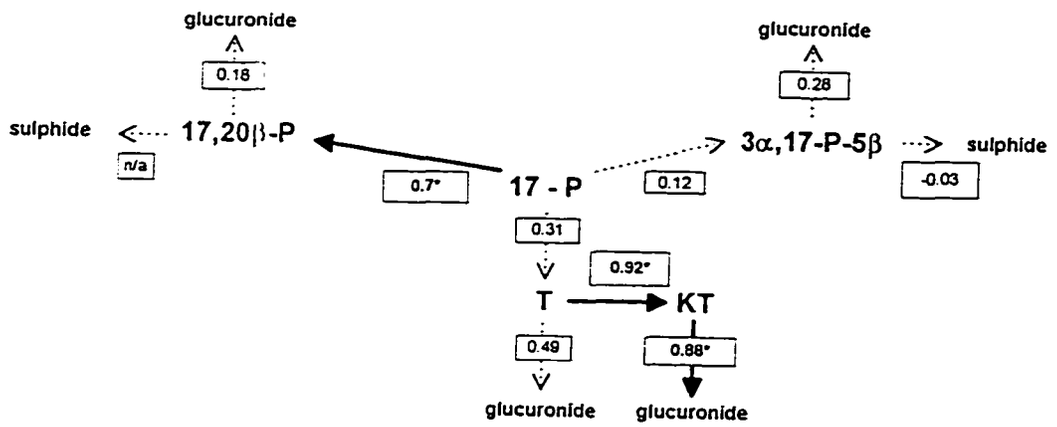
The profiles of reproductive steroids that I observed in relation to maturation of herring in the present chapter indicate that this species generally fits the model of teleost oocyte maturation proposed by Nagahama (1987, 1994) and male maturation proposed by Fostier et al. (1987) but with some distinctive aspects.

Female maturation:

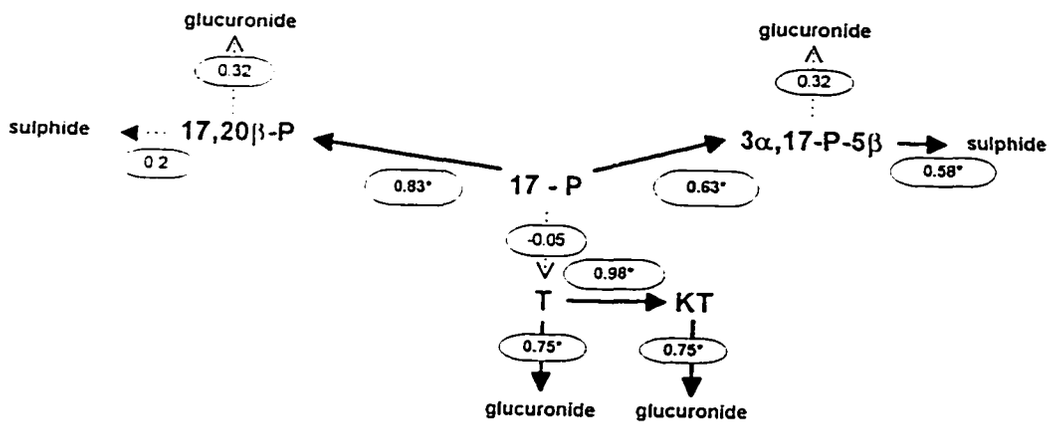
Ovarian histology of the stage IV female herring of our study showed that the maturing oocytes in these fish ranged from late vitellogenic to

Fig. 16. Correlation between plasma levels of metabolically related steroids in A) maturing male fish (pre-milt producing stages IV and V; n = 12) and B) ripe male fish (milt-producing, stage VI; n = 27). Arrows indicate metabolic pathways, whereas numbers in boxes indicate bivariate correlation coefficients between levels of the respective steroid forms. Significant correlations (α = 0.05) are indicated by an asterisk (*) and a solid line of the arrow. Abbreviations of steroid forms as in Fig. 15.

A) Maturing male fish (stages IV & V)



B) Ripe male fish (stage VI)



fully mature, whereas the relatively high mean gonadosomatic index (GSI), and the high level of T glucuronide at this time suggest that vitellogenesis was mostly completed by the end of February. This may explain the lack of significant levels of estradiol in our samples. T peaks in most teleosts after vitellogenesis ends, probably due to continued synthesis while conversion to estradiol stops (see Nagahama, 1994). The same may be true for herring, suggesting that females that had completed vitellogenesis (stage V by Bowers and Holliday's (1961) criteria) were classified as late stage IV fish in our study. The negative correlation between GSI and levels of T glucuronide in ripe females is likely to be an indicator of the extent of maturation a fish has reached, in that GSI increases in "ripe" fish are mostly due to egg hydration associated with final maturation, whereas elevated T glucuronide levels may be carried over from the end of vitellogenesis.

At the end of vitellogenesis, final maturation of oocytes in teleosts occurs in association with the production of a maturation-inducing steroid (MIS), and the sensitization of the oocytes to the MIS (Nagahama, 1994). Levels of 17-P; 3α,17-P-5β and 17,20β-P increased in the plasma of herring in the peri-ovulatory period, indicating that these are the steroids associated with oocyte maturation. 17,20β-P is the most likely MIS candidate of these three progesterones, despite its relatively low plasma levels. The other two steroids are unlikely candidates because: 1) maturational effects of 17-P have generally been shown to be mediated through conversion to 17,20β-P (Scott and Canario, 1987) and 2) 5β-reduced pregnans have been found to have a maturational effect only in one species of catfish, and this effect was secondary to 17,20β-P (Upadhyaya and Haider, 1986). A sulphated 5β-reduced metabolite of 17,20β-P is also one of the most abundant steroids during oocyte-

maturation in the plaice *Pleuronectes platessa*, but is believed to be present primarily as a result of rapid metabolism of the true MIS, 17,20β-P (Scott et al., 1996). Sulphated 3α,17-P-5β is also the only steroid found to be correlated with gonadosomatic index in maturing females, presumably reflecting metabolism of excess 17-P during this period. The correlation between levels of 17-P and both of the other progesterones is very high in these fish, also suggesting that 17-P is being rapidly metabolised. Clearly the maturational effects of 17-P, 17,20β-P, and 3α,17-P-5β in the herring need to be tested to understand the dynamics of this steroidal system, though some further comparisons with other species are illustrative.

17,20β-P has been found to be the most potent MIS *in vitro* in a variety of species of catfish (Haider and Rao, 1992) and flatfish (Canario and Scott, 1990a) despite low or nondetectable plasma levels. Canario and Scott (1990a,b) argue that only minute amounts and short exposure to the MIS are needed to elicit maturation in oocytes and that high levels of free 17,20ß-P found in some salmonids is due to the absence of both conjugation and 5ß-reduction. The low levels of free 17,20ß-P in maturing herring could result from a combination of rapid glucuronation and marked competitive conversion of 17-P to 3α,17-P-5β, as suggested by the relatively high plasma levels of 3α,17-P-5ß and glucuronated 17,20ß. Canario and Scott (1990a,b) suggest that low levels of 17,20ß-P may be particularly important to serial spawning fish to ensure localized oocyte maturation in the ovary, but this is an unlikely function in the herring, since oocytes appear to ovulate synchronously (Bowers and Holliday, 1961).

I also measured 11-DOC, 17,20α-P, and 17,20β,21-P in the plasma pools of herring. However, only 11-DOC showed a seasonal profile in the

herring consistent with a maturational role. 11-DOC is produced by interrenal cells of Atlantic herring in the presence of excess 17-P (Sangalang et al., 1972) and in ovarian tissue of the tobinumeri-dragonet (Asahina et al., 1992). However, when maturational effectiveness *in vitro* has been tested, it has invariably been found to be lower than that of 17,20β-P or similar MIS candidates (Scott and Canario, 1987; Asahina et al., 1992).

Scott and Canario (1987) suggest that high levels of MIS and MIS-related metabolites in the blood indicate a pheromonal role for these steroids in addition to their MIS-related function. Indeed, 17,20β-P and its metabolites are pheromonal in various cyprinids (Stacey and Sorensen, 1986), as is the glucuronide of 3α,17-P-5β in the African catfish (Resink et al., 1989). The most abundant steroids in the peri-ovulatory herring are 17-P, 3α,17-P-5β, and T glucuronide. I reported earlier that 17-P and T glucuronide have no activity as spawning pheromones in the Pacific herring (Carolsfeld et al., 1992), and also found that a cocktail of 10^{-4} M 3α,17-P-5β and 3α,17-P-5β glucuronide did not elicit spawning (0/7 fish). However, activity of these compounds as maturational or priming pheromones has not been tested.

Female holding and spawning:

Holding of ovulated eggs before spawning, as occurs in the herring, also is distinctive of salmonids (Huet, 1986) but is not as prolonged. In the present study, ovulated eggs were held in the herring for over two months, suggesting that either there is some mechanism for maintaining the viability of these gametes in the absence of blood supply or ovulation is incomplete. Plaice ovulation, for example, is preceded by a period of loose follicular connections, during which fully hydrated and fertilizable

eggs can be physically jarred loose and stripped (Scott and Canario, 1990). However, several lines of evidence suggest that herring eggs, unlike those of the plaice, are fully ovulated while being held: 1) histological evidence of retained follicular connections has not been found (Bowers and Holliday, 1961; Gillis et al., 1990a,b; and the present chapter); 2) pre-vitellogenic oocytes in the ovarian lumen were absent, but would be expected if oocytes had been artificially ovulated by the sampling protocol; 3) a gradual reduction in viability of eggs and larvae with extended holding has been reported (Hay, 1986), though no histological evidence of deterioration of eggs was found in the present research; 4) adhesiveness of eggs develops only after disruption of the follicular envelope (Gillis et al. 1990a); and 5) the oviduct of herring produces a secretion after ovulation that may function in the maintenance of ovulated eggs (Bowers and Holliday, 1961). Particularly "runny", easily expressed gametes are nevertheless a characteristic of actively spawning Pacific herring and are rarely seen otherwise (Hay, 1985). These fish correspond with stage VI of Hay's (1985) scale, but were combined with the remaining "ovulated" fish in our study according to Bowers and Holliday's (1961) criteria. The basis of this increased "runniness" remains to be determined, but may be indicative of an additional maturational step beyond ovulation (Hay, 1985). However, I found that during deep anesthesia (stage 5, Summerfelt and Smith, 1990) with tricaine (350 ppm), fish of both stages have equally "runny" eggs, in a reversible manner. This suggests that runniness is not a reflection of the level of ovulation, but rather may result from relaxation of sphincter or oviduct muscles. Spawning of females was synchronous and complete in our study, but I did not find steroidal indicators of a further maturational step before spawning.

Male maturation and start of milt production:

Male GSI did not increase as markedly at the start of the sampling period in my study as in that observed by Gillis et al. (1990b), suggesting that the male fish I studied had already completed much of the rapid gonadal growth preceding milt production. This rapid gonadal growth in stage IV male herring probably coincides with spermiogenesis (conversion of spermatids into spermatozoa) (Bowers and Holliday, 1961), but probably also includes spermiation (see below). 11-KT is elevated coincidentally with T in these fish and may also be involved in spermiogenesis, as Fostier et al. (1987) and Borg (1994) suggest. In contrast, the peak 11-KT levels in some fish species follows the T peak, coinciding with maximum milt production in some cases (Kime and Manning, 1982), but probably primarily involved in the development of secondary sexual characteristics (Baynes and Scott, 1985). 11-KT is not likely to serve this function in herring, since this species does not possess secondary sexual characteristics. GSI of maturing males is most clearly correlated with levels of glucuronated T, suggesting that T is the steroid most related to this phase of growth. However, the correlation between levels of T and T glucuronide was low in "maturing" fish that I sampled, as was the correlation between 17-P and T. This may also indicate that the fish I sampled had mostly completed gonadal growth and were initiating milt production, with a concomittant switch to production of progesterones rather than androgens (see below).

The initiation of milt production in the herring is accompanied by a marked increase in progesterones, presumably due to an increased conversion of 17-P to progesterones rather than to T. 17,20β-P was shown by Ueda et al. (1985) to coincide with gonadotropin-induced milt production (termed "spermiation" in their paper), and was more potent in

inducing this milt production than androgens in both amago salmon and the goldfish. 11-KT is generally the predominant androgen in salmonids during maximum sperm production (Kime and Manning, 1982), but Baynes and Scott (1985) report that initiation of "spermiation" coincides with the peak of T. I found that T is high in herring preceding milt production whereas the highest concentrations of progesterones are present in fish that have recently started producing milt and levels of 17-P and 17,20β-P are correlated with expressible milt volume. Progesterones, rather than T, are thus likely to be important for the start of milt production. Conjugated 17,20ß-P and 3α,17,21-P-5 in the milt, plasma, and urine of Pacific herring and conjugated 3α,17,21-P-5 in the milt (which may have been 3α,17-P-5) have been reported earlier (Scott et al., 1991a,b). These probably reflect the elevated levels of these steroids during milt production, though the anomolous high level of conjugated 17,20ß-P in the urine of "spent" male herring (Scott et al., 1991a) remains unexplained, as does the poor correlation between levels of conjugated and free progesterones in male fish in my study.

Schoonen (1986) correlated plasma levels of 3α,5ß reduced progesterones with spawning readiness in the African catfish and I found the same with Pacific herring (Chapter 4), but so far no maturational effect of these steroids in male fish has been reported. I found a good correlation between levels of 17-P and 3α,17-P-5β in ripe but not maturing males, suggesting that the role of this steroid is more pronounced in ripe fish. However, the role of these steroids in the herring remains to be elucidated.

Male holding and spawning:

The extended period of milt production observed in the herring of this study is not unusual amongst fish species (see Harvey and Carolsfeld, 1993). However, the physiological status of the testes during this time is not clear. Bowers and Holliday (1961) report that spermatozoa are present in the testicular lumen of the Atlantic herring throughout gonadal maturation (stages II to V) and are all in the lumen by stage VI (i.e. spermiation is ongoing throughout testes recrudescence and complete when milt production starts). My histological examination of testes of milt- producing Pacific herring likewise found that spermiation was complete, indicating that a process other than spermiation initiates milt production in herring. Milt hydration is likely to be one of the factors involved in milt production (Harvey and Carolsfeld, 1993).

Levels of free 17-P, 17,20β-P and 3α,17-P-5ß increased at the time of milt production, suggesting that these hormones are involved in spermatozoan mobilization. The rise in 17-P may be indicative of gonadal steroidogenesis, whereas the rise in either 17,20β-P (produced by testicular tissue and/or the spermatozoa, Ueda et al., 1984) or 3α,17-P-5ß may indicate spermatozoan mobilization. Because partially spent males were common as the season progressed, episodic spawning or milt release by some individuals appears likely.

Despite the observed association between progesterones and expressible milt volume, I have no clear indication that steroids are involved in controlling spawning behaviour of male herring. Androgens have been implicated in male reproductive behaviours of some fish species (e.g. Liley et al., 1993; Pankhurst and Barnett, 1993), possibly after aromatization in the brain (Callard et al., 1990). Liley et al. (1993) and Pankhurst and Barnett (1993) also report elevated progesterone

levels in association with reproductive behaviour, but the role of these steroids in control of behaviour is still unknown.

Steroid conjugation:

The gonads are a major site of glucuronation and sulfation for teleost reproductive steroids (Scott and Vermeirssen, 1994). However, the level of conjugation varies among species, and the specificity of glucuronyl and sulfyl transferases remains controversial. In the herring, glucuronation is the principal type of steroid conjugation resulting in conjugation ratios (concentration of glucuronated form / free form) of 50-150 for androgens and 10-50 for 17,20ß-P. In comparison, salmonid and dab glucuronation ratios of the same steroids are less than 1, and those of plaice are 2-8. The glucuronation ratio of 3α,17-P-5β (1-4 in females and 2-25 in males) was nearly a magnitude lower than that of the other steroids (10-250). 3α,17-P-5β may compete with another steroid for glucuronation (possibly 17,20β-P), as evidenced by the opposite seasonal trend in glucuronation ratios to that of 17,20ß-P (Figs. 13, 14).

Plasma levels of sulphated testosterones are negligible in the herring, and levels of sulphated progesterones are quite low. Testosterone sulphation has also been found to be very low in other fish species, whereas low levels of sulphated progesterones may be due to rapid renal elimination (Scott and Vermierssen, 1994). However, the low correlation between levels of sulphated and free 17,20β-P in all groups of fish tested, compared to significant correlations between these two forms of 3α,17-P-5β in all but the maturing males remains to be explained, as does the low correlation between glucuronated and free forms of both progesterones in all groups of male fish.

Pheromonal control of spawning and delayed spawning in captivity:

Partially spent male, but not female, fish were found in the net pens several weeks before general spawning of the school. Thus, some males in the net pen spawn or leak milt intermittently before the rest of the school spawns. I searched for spawned eggs, but did not find them in the net pen prior to the general spawning, also indicating that female fish had not spawned. Partially spent males are also common in wild schools as spawning approaches (Hay, 1985), indicating that imtermittent partial spawning of male fish is not limited to the captive situation. The significance of this finding is that milt, which theoretically contains the spawning pheromone, must have been present in the water of the net pen at various times at least several weeks before mass spawning. The fish that I studied were also exposed to milt from an adjacent spawning wild school shortly after final maturation, but did not spawn. A majority of female and male fish are responsive to the spawning pheromone present in milt throughout most of the ripe holding period (Chapter 2), but the presence of spawning pheromone in the small quantities likely to have occurred during the season were clearly not sufficient for triggering mass spawning. Additional environmental cues, such as availability of appropriate spawning substrate (Kreiberg et al., 1982), may be needed for effective spawning. These cues were probably deficient in the captive situation I used and may have contributed to the prolonged delay in spawning.

Evolutionary implications of herring reproductive physiology:

As a fish representative of a basal teleost lineage (Nelson, 1984), aspects of the reproductive physiology of the herring may help provide insights to the evolution of steroid function and metabolism of vertebrates.

Distinctive characteristics of herring reproduction include 1) low levels of free 17,20β-P, the putative maturation-inducing steroid (MIS) in females; 2) high levels of 17-P and its 5β-reduced metabolite, 3α,17-P-5β in both sexes; 3) high levels of T in female fish; and 3) similar rather than disparate 11-KT and testosterone levels in males.

17,20β-P has been implicated as the MIS in a large variety of fish (Scott and Canario, 1987), including an osteoglossomorph (Pankhurst et al., 1986), and is thus probably the primitive teleostean MIS. This steroid results from β-hydroxylation of 17-P. In contrast, the α-hydroxylated molecule (17,20α-P) is the principal metabolic product of 17-P in the human (Manire et al., 1995), but is of uncertain function. The precursor to 17-P, progesterone (P) without hydroxyl groups, is the progesterone most studied in vertebrates other than teleosts. This progesterone is important for maintenance of pregnancy in elasmobranchs (Manire et al., 1995) and humans (Milgram , 1990). Diversification of the MIS in teleosts appears to be a derived characteristic. Confirmed maturation-inducing steroids other than 17,20β-P occur primarily in more recently evolved perciform species (Thomas, 1994), in some cases in addition to a continued MIS role of 17,20β-P (Asahina et al., 1992; King et al., 1994).

Low circulating levels of the 17,20β-P MIS have been reported from some catfish species and several species of the more recently evolved Pleuronectiformes (see Canario & Scott, 1990b), whereas high levels have been reported from the more basal cyprinids (Santos et al., 1986) and salmonids (Scott et al., 1983). Some recently evolved perciform species have high circulating levels of a different MIS, 17,20β,21-trihydroxyprogesterone (17,20β,21-P), a metabolite of 17,20ß-P, which is itself present in low amounts (Thomas, 1994). Canario and Scott (1990a,b) have suggested that high levels of the MIS are unnecessary for gonadal maturation and

deleterious for serial spawning fish. These authors postulate that high levels of MIS, when present, serve for pheromone production rather than maturation. Likewise, they suggest that MIS-related steroids in species with low MIS levels are a rich source of pheromones, and observe that enzymes for steroid conjugation and 5α- or ß- metabolism are likely to be missing in some salmonids with high MIS levels. However, the metabolic mechanisms that result in reduced MIS levels appear to be diverse, suggesting that the low MIS condition has evolved several times.

Low levels of the putative MIS in herring remains to be confirmed by more frequent sampling than I was able to carry out, but chronic high levels as occur in the salmon are clearly not present. The reason for the low levels is unknown, because female Pacific herring are not known to be serial spawners. The probable mechanism for maintaining low levels appears to be competitive conversion of 17-P into 3α,17-P-5β, a mechanism that may also be present in the African catfish, judging by the high plasma levels of 3α,17-P-5β in this species (Schoonen, 1986). It is possible that this mechanism was lost (or better controlled) during evolution, leading to high MIS levels in some fish and alternate control by metabolism of 17,20β-P in others. 17,20β,21-P, the MIS in some perciformes, could initially have been one of these metabolites, subsequently becoming a MIS with high circulating levels.

The presence of relatively high levels of testosterone in the blood of females appears to be a common feature of teleosts (Nagahama, 1994), elasmobranchs (Manire et al., 1995; Koob et al., 1986; Tsang & Callard, 1987) and some reptiles (Brown et al. 1994; McPherson et al., 1982). In most of these cases, testosterone is thought to function primarily as a precursor to estrogens, and elevated testosterone in the blood are believed to reflect continued high synthesis rate of testosterone when rates of aromatization to estrogen are reduced. In contrast, levels of testosterone in the blood of

human females are not elevated and do not change with estrogen production (Ganong, 1987; Milgram, 1990). A possible reason for this difference is that testosterone and estrogen are produced in different cells in the majority of fish (Nagahama, 1994), whereas they are produced by the same cell in humans (Milgram, 1990). High levels of progesterone in the blood of humans has likewise been attributed to a two-cell model where the steroid substrate (progesterone) is produced in granulosa cells and converted to testosterone and estrogen in thecal cells. Thus, in both fish and humans steroids that need to pass from one cell type to another to complete a metabolic pathway are most likely to be found in the blood.

The high levels of 17-P in the blood of mature herring and the unexpectedly low correlation between T and 17-P may also be due to the difference between the site of steroid conversions. 17-P and testosterone are both produced within thecal cells of many fish, whereas 17-P is converted to the MIS in adjacent granulosa cells during final maturation (Nagahama, 1994). I suggest that 17-P is secreted from thecal cells only during final maturation, and thus appears in the blood at this time. Intracellular conversion of 17-P to testosterone may continue in the thecal cells during final maturation, but not necessarily in a manner that is correlated with rates of secretion of 17-P. This model explains the low levels of 17-P in the plasma of fish while testosterone is high, but also suggests that the switch from C_{19} to C_{21} steroids during final maturation of fish may not only involve changes in activation of the enzyme systems generally considered (e.g. Nagahama, 1994), but may also involve enhanced "secretion" mechanisms for 17-P. However, not all fish use a two-cell mechanism for steroid production during final maturation (Lin et al., 1991), and plasma levels of steroids in these fish need to be investigated.

Testosterone and 11-KT are the principal bioactive androgens in the reproductive physiology of male teleosts (Kime, 1980), but their respective roles remain equivocal. Early stages of spermatogenesis appear to be generally associated with testosterone (Fostier et al., 1987), whereas later stages are associated with 11-KT (e.g. Scott and Sumpter, 1989; Campbell et al., 1976) or the 11-KT precursor, 11-hydroxytestosterone (Idler et al., 1971). Borg et al. (1989) and Vermeulen et al. (1992) have suggested that 11-keto and 11-hydroandrostenedione (alternate metabolites of the testosterone precursor) are important to male reproduction in stickleback and catfish, respectively. Dihydrotestosterone (5α-reduced T), the testosterone metabolite of principal bioactive importance to mammalian reproductive physiology and secondary sexual characteristics (Milgram, 1990), also appears to play some role in reproduction of elasmobranchs (Manire et al., 1995) and lampreys (Katz et al., 1982).

Our results with herring suggest that testosterone and 11-KT are the principal androgens in males of this species, as in other teleosts, but little evidence was found to clearly distinguish their respective roles. Herring possess an apparently primitive school spawning strategy, which involves neither pair formation nor secondary sexual characters (Carolsfeld et al., 1992). This implies that maturational effects of testosterone metabolites, such as 11-KT-induced secondary sexual characters, evolved together with more complex spawning strategies, or were lost in herring.

Future comparative studies, including more detailed work on the herring, should help to clarify the evolutionary trends in steroidal mechanisms of teleost reproductive physiology and their relationship to pheromonal communication.

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CHAPTER 4. PLASMA STEROIDS DISTINCTIVE TO HERRING RESPONSIVE TO THE SPAWNING PHEROMONE[3]

ABSTRACT

A spawning pheromone in the milt (semen) and testes of the Pacific herring, *Clupea harengus pallasi*, is thought to facilitate school spawning of this species. I found that responsiveness to the spawning pheromone was variable amongst ripe fish (milt-producing or ovulated). Measurement of five principle reproductive steroids in the free form and five steroids in conjugated forms in the plasma of male fish early in the spawning season (newly-ripe fish) showed that elevated plasma levels of 3α,17α-dihydroxy-5ß-pregnan-20-one (3α,17-P-5β) and 17α-hydroxyprogesterone (17-P) coincided with responsiveness to the spawning pheromone in these fish; levels of other steroids did not differ. In contrast, responsiveness to the pheromone by female fish later in the spawning season (ripe-and-holding fish) coincided with lower levels of glucuronated 17α,20ß-dihydroxyprogesterone (17,20ß-P) and a lower gonadosomatic index. I suggest that these differences indicate a more advanced mature reproductive state in the responsive individuals amongst both the newly-ripe male and the ripe-and-holding female fish. I found no differences in the level of cortisol in the blood of the herring that could be correlated with differences in pheromonal responsiveness. I conclude that differences in responsiveness to the spawning

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[3] A version of this chapter has been accepted for publication in *Hormones and Behavior* with co-authors A.P. Scott (Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research, Fisheries Laboratory, Lowestoft, Suffolk, NR33 OHT, England) and N.M. Sherwood (Biology Dept., UVic). Most of the steroid analyses were carried out by J. Carolsfeld at Lowestoft with the assistance of Dr. Scott.

pheromone coincide to some extent with levels of reproductive maturation but probably not with recent stress.

INTRODUCTION

Pacific herring *Clupea harengus pallasi* spawn in large schools of fish without evident physical interaction between individuals. A pheromone present in the milt of this species is thought to facilitate this type of "school spawning", by inducing spawning behavior of ripe individuals of both sexes (Stacey & Hourston, 1982; Sherwood, et al., 1991; Carolsfeld et al., 1992). Working with small groups of captive ripe herring, Stacey and Hourston (1982) demonstrated that the pheromone induces spawning behavior within minutes of exposure and thus they provided one of the clearest examples of a reproductive-releaser pheromone in fish. Subsequently, this pheromone was partially characterized with a bioassay in which the extension of the gonadal papilla and spawning behavior in response to pheromonal stimulation was observed in individual herring in small aquaria (Sherwood et al., 1991).
I described a further characterization of the behavior of the fish in the aquarium assay and larger tanks in Chapter 2. An enigmatic observation in this work is that up to one third of apparently similar individuals do not respond to the pheromone in the aquarium assay. The responsiveness to reproductive pheromones at a particular maturational level (ripe) has been reported for herring (Stacey and Hourston, 1982; Sherwood et al., 1991) and female African catfish (Resink et al., 1987). Reproductive pheromones of fish that have been identified are almost exclusively reproductive hormones or their metabolites that are excreted at particular times during maturation (Stacey and Sorensen, 1991). Schoonen and Lambert (1986) have suggested that 5ß-reduced C_{21} steroids in ripe male

catfish are the source of pheromones associated with spawning. However, this chapter and Chapter 2 are the first to investigate individual variability of pheromonal responsiveness. In this chapter I study the relationship between steroid hormone levels in the blood of ripe herring and pheromone responsiveness. My hypothesis is that pheromonal responsiveness is the result of differences in reproductive maturation and/or stressful events associated with the captive environment that are reflected by levels of steroid hormones in the blood.

MATERIALS AND METHODS

Fish

Adult herring were captured by purse seine in Trincomali Channel, B.C. in mid-February, 1994, prior to ovulation and spermiation and transported by a live holding barge to Departure Bay, Nanaimo, B.C. The fish were maintained in 20 x 30 x 5m deep net pens at the Experimental Fish Farm of the Pacific Biological Station (Department of Fisheries and Oceans), without artificial feeding, until after spawning had occurred in mid-May to early June. Stocking density was about 1 ton of fish per net pen. After the majority of fish had ovulated or commenced producing milt in early March, mixed sex batches of 100-200 individuals were transferred to 600 l circular holding tanks, 2m in diameter. These tanks were supplied with 11-13 °C flowing sea water pumped from Departure Bay. Fish were taken at random from the holding tanks within one week, sexed by the presence of milt or eggs expressed with gentle abdominal pressure, and transferred in lots of 25-30 fish of one sex to 60 l buckets in a laboratory supplied with running sea water. Fish were netted from these buckets and transferred to assay aquaria within 1 hr.

Classification of responsiveness to the pheromone

An initial study was carried out with fifteen recently matured male fish in early March, 1992 ("newly-ripe" fish). These were sexed and taken from the holding tanks as described above five days after transfer from the net pens. Classification of pheromonal responsiveness was carried out in "group assays" comparable to those described in Chapter 2. For these assays, groups of five fish were randomly netted from the holding bucket and placed in freshly cleaned 30 l glass aquaria lighted from the top with a 25 watt incandescent light. The fish were then observed for a 15 minute acclimitization period from a darkened room, and those that showed prolonged extension of the gonadal papilla or substrate spawning behavior ("spontaneous" fish) were carefully removed by hand before release of milt. These fish were eliminated from this first experiment. After the acclimization period, 1 ml of fresh milt diluted 1:1 with sea water was applied to the center of the aquarium by pipette. A positive pheromone response occurred within 3 minutes, consisting of an extension of the gonadal papilla and initiation of spawning. The fish were thus classified as "responsive", or "non-responsive" based on their behavior in this assay. The fish in the assay are likely to have encountered adequate stimulus of pheromone to permit classification based on the facts that: 1) the stimulus used is approximately 50 fold that of threshold concentrations, 2) fish passed through the center of the aquaria about every 10 s, and 3) the stimulus cloud took about one minute to dissipate throughout the aquarium (Chapter 2).

A second experiment was carried out in late April, 1994, with fish taken from a net pen in which individuals had completed maturation in early March, as had the fish in 1992, and spawned shortly after the fish of

this experiment were removed (Chapter 3). These "ripe-and-holding" fish of both sexes were taken from the holding tanks five days after transfer from the net pen, but were tested for responsiveness in individual assays described in Chapter 2, rather than the group assays. Briefly, fish were placed into individual 60 l aquaria, and, after an acclimitization period during which "spontaneous" fish were identified and sampled, two pheromonal stimuli of 0.25 ml of fresh milt diluted 1:1 with sea water were pipetted onto the nares of the fish at an interval of about three minutes. Seven such aquaria were used in parallel. Fish responsive to the pheromone were sampled after clearly responding to the pheromone (up to 3 minutes after the final stimulus), whereas non-responsive fish were sampled 5 minutes after the final stimulus. No spontaneous female fish were encountered during this experiment.

Blood sampling and morphological characterization

Fish were killed by a blow to the head immediately after removal from the assay aquarium, either before (spontaneous fish) or within 5 minutes of the pheromonal stimulus (responsive and non-reponsive fish). Blood was sampled from the caudal sinus with heparinized needles immediately after death. Subsequently, total weight, gonad weight, length, expressible milt volume (males only), and spermatocrit (males only) of each fish were determined. Spermatocrit was determined in triplicate with milt that was gently extruded with abdominal pressure from freshly killed fish and aspirated directly from the gonadal pore with hematocrit tubes. Contamination of urine was avoided by visual inspection of the milt droplet, since urine was of a distinctive yellowish colour and not homogeneously mixed with the milt. The hematocrit tubes were centrifuged for 15 minutes in a hematocrit centrifuge before

measuring the proportion of packed sperm cells. The average value of the three determinations per fish was taken as the spermatocrit value.

Steroid analysis

Plasma samples were semi-purified on C_{18} Sep-Pak cartridges (Waters, Millford, MA) prior to steroid analysis to eliminate interfering non-polar lipids. This was done by loading 100 µl of plasma, diluted to 0.5 ml with distilled water, onto a Sep-Pak cartridge that had previously been primed with methanol and rinsed with water (as per manufacturer's instructions). The cartridge was then rinsed with a further 2 ml of water, and the steroids eluted with 4 ml of methanol. The methanol was removed under a stream of nitrogen at 45°C and the resulting residue was reconstituted with 100 µl of distilled water.

Free and conjugated steroids in the purified plasma were extracted as described by Scott and co-workers (Scott et al., 1984; Scott & Sorensen, 1994) and as described in Chapter 3. Briefly, free steroids were extracted directly from the 100 µl of reconstituted Sep-Pak-purified plasma with 4 ml of ether. The aqueous residue was solvolyzed with trifluoroacetic acid (TFA)/ethyl acetate (1.4:100) overnight at 45°C to free sulphated steroids, which were then extracted with ether after removing the TFA/ethyl acetate under a stream of nitrogen. Finally, glucuronated steroids were freed from the aqueous residue of the solvolyzed plasma by digestion overnight with *Helix* aryl sulphatase/ glucuronidase (Sigma) at 37°C in an acetate buffer (pH 8.3). The freed steroids from this digestion were also extracted with ether. All ether extracts were dried down in a 45°C water bath and reconstituted in 1 ml of assay buffer (0.1 M phosphate buffer with 0.1% bovine serum albumin (BSA) and 0.1% sodium azide) and stored frozen in this buffer until analysis.

Reproductive steroids to be measured were selected on the basis of previous work (Carolsfeld et al., 1996; Chapter 3). Testosterone, 11-keto testosterone (11KT), 17-hydroxyprogesterone (17-P), 17α,20β-dihydroxyprogesterone (17,20β-P), and 3α, 17α-dihydroxy-5β-pregnan-20-one (3α,17-P-5β) were measured in the free steroid fractions. The same steroids except for 17-P were measured in the glucuronated fractions, and 17,20β-P was measured in the sulphated fractions. Measurements were carried out with radioimmunoassays as described by Scott et al. (1984) and Canario & Scott (1990). Characterization and evaluation of the assays is described in these papers. The assay used to measure 3α,17-P-5β detects several 5β reduced pregnanes (Canario & Scott, 1990), but the immunoreactivity found in herring blood was determined to be primarily 3α,17-P-5β by high pressure liquid chromatography (Fig. 9; Chapter 3). The complete set of plasma samples was assayed at one time for each steroid to avoid inter-assay variability.

Cortisol was measured with a miniaturized assay based on Scott et al. (1984). Assay tubes in this procedure contained 50 μl of sample and 50 μl of the antibody-tritiated cortisol reagent. The reagent mixture consisted of 100 μl of antibody suspension (Endocrine Sciences, Calabasis Hills, Calif., F3-314 antibody, Lot 345-102280, diluted 1:100 in a 0.5 M phosphate buffer) added to approximately 320,000 counts of 1,2,6,7-^{3}H cortisol (New England Nuclear/Dupont, Markham, Ont.) in a volume of 8 ml of 0.5 M phosphate buffer (pH 6) containing 0.1% BSA. Standards were prepared to contain 500 - 1.95 pg cortisol/50 μl in a series of doubling dilutions. The assay was incubated for ninety minutes at room temperature, then placed in an ice bath, where unbound steroids were precipitated with 250 μl of a 0.5 % dextran-5% charcoal suspension in 0.05 M phosphate buffer with 0.01% gelatin. After centrifugation, the

supernatant was decanted into 1.5 ml polypropylene Eppendorf vials and combined with 1 ml of Scintisafe 50 (Fisher Scientific) scintillation fluid. The Eppendorf vials were placed inside standard scintillation vials for counting with a Beckmann scintillation counter (duplicate four minute counts). Mean replicate coefficient of variability within this assay was typically 2.6% (n=30), with a range of 0.04 to 13%. Levels of conjugated cortisol were not measured.

Statistics

Morphological information and steroid levels of the different response classes were compared within each sampling date and sex with an Anova for the male ripe-and-holding group and Student's t test for the other groups (Zar, 1974). A nominal α level of 0.05 was used, corrected within each comparison between groups of fish with Bonferroni's correction factor for multiple comparisons (Rice, 1989). Values are expressed as means ± standard errors of the mean (S.E.) throughout the text unless otherwise indicated.

RESULTS

Morphological characteristics

I found that the gonadosomatic index of ovulated-and-holding female fish responsive to the pheromone was significantly lower than that of similar fish that were not responsive to the pheromone (corrected α=0.017). Expressible milt volume of responsive newly-ripe male fish to was greater than that of non-responsive fish of this sampling date only for an uncorrected α level of 0.05, and not for an α level corrected for the multiple comparisons between these two groups (corrected α=0.01). No

other differences in length, weight, gonadosomatic index, milt volume, or spermatocrit was detectable between the response classes of the two sampling dates (Table 9).

Relation of steroid hormones to pheromone responsiveness

Plasma levels of 17-P and free 3α,17-P-5β of newly-ripe male fish were significantly higher (Bertollini-corrected $\alpha = 0.005$) in responsive individuals than in non-responsive fish (Table 10). The level of glucuronated 17,20ß-P was significantly lower in responsive ripe-and-holding female fish than in non-responsive fish of this group at an α-level of 0.05, but not at a Bertollini-corrected α-level of 0.01. Levels of the other steroids measured did not differ significantly between responsive and non-responsive fish.

Levels of plasma steroids of the fish sampled in March of 1992 were very similar to those of similar fish sampled in 1994 (Carolsfeld et al., 1996; Chapter 3), suggesting that the results from the two sampling periods I used in the present paper are comparable.

DISCUSSION

I found that, amongst the reproductive factors investigated, elevated plasma levels of 17-P and 3α,17-P-5β were significant correlates of pheromonal responsiveness in newly-ripe male fish. Milt volume may also distinguish these fish, but possibly only with a larger sample size. The application of the Bertollini correction to the α-level of multiple comparisons is somewhat subjective (Rice, 1989), and may thus obscure factors that are biologically important. Other morphological indicators commonly used to describe the reproductive condition of fish, including

Table 9. Morphological characteristics of herring with different responsiveness to the spawning pheromone at different times during the spawning season (Means ± S.E.).

Maturity class (sampling dat	Pheromone response class[1]	Total weight (g)	Total length (cm)	GSI[2] (%)	Spermato-crit[3]	Milt vol (ml)	n
MALES							
Newly spermiating (Mar. 5, 1992)	**+ve**	**80.6** ± 2.9	**197.2** ± 3.0	**16.5** ± 0.8	**0.95** ± 0.00	**1.9** ± 0.7	5
	-ve	**75.4** ± 7.2	**192.7** ± 6.5	**17.0** ± 1.9	**0.87** ± 0.07	**0.7** ± 0.2	10
Spermiating & holding (Apr. 21 & 22, 1994)	**+ve**	**62.3** ± 6.2	**178.0** ± 4.0	**23.2** ± 2.1	**0.93** ± 0.02	**1.8** ± 1.4	3
	-ve	**88.8** ± 10.1	**202.8** ± 11.5	**22.3** ± 1.2	**0.92** ± 0.02	**1.2** ± 0.3	5
	Spont.	**94.3** ± 17.6	**209.8** ± 10.2	**18.2** ± 3.4	**0.89** ± 0.03	**1.8** ± 0.8	4
FEMALES							
(Ovulated & holding Apr. 21 & 22, 1994)	**+ve**	**85.9** ± 8.8	**190.3** ± 7.3	**21.9** * ± 0.8	n/a	n/a	6
	-ve	**95.7** ± 12.5	**195.7** ± 9.9	**26.7** ± 1.1	n/a	n/a	6

[1] +ve = responsive to pheromone, -ve = not responsive to pheromone stimulus, spont = spontaneous spawning without overt pheromone stimulus.

[2] gonadosomatic index = weight of gonads / weight of intact fish

[3] spermatocrit = propotionate volume of packed sperm cells in milt

* significantly different from levels in non-responsive fish (Bonferroni-corrected α=0.01 for males, 0.017 for females)

these hormones correspond to reproductive maturation. Levels of the reproductive steroids that were measured in the present study, other than 17-P and 3α,17-P-5ß, were generally comparable in newly ripe fish to those found in the earlier study in male fish of this maturity. The fish of the present study thus are likely to be a valid representation of newly-ripe male herring. The differences in plasma levels of 17-P and free 3α,17-P-5β in fish of this maturity but different responsiveness to the pheromone are consistent with slight differences insexual maturity when compared to our earlier results (Carolsfeld et al., 1996; Chapter 3). The levels of these steroids in pheromone-responsive fish are comparable to those of ripe male fish, whereas the lower levels in non-responsive fish are most comparable to levels of less mature fish. Levels of these steroids remained elevated for over one month after the start of milt-production (Carolsfeld et al., 1996; Chapter 3), so non-responsive fish are less likely to be of more advanced maturity. The greater expressible milt volume of responsive fish (Table 9) also suggests that these fish are more mature than the non-responsive fish.

However, glucuronated levels of 3α,17-P-5β, although quite variable in all groups, were as high in non-responsive newly-ripe male fish as in responsive fish of this group. Plasma levels of conjugated steroids were studied in addition to the more commonly investigated free steroids because levels of the free form of key steroids in some fish are very low (Scott and Vermierssen, 1994), including the herring (Carolsfeld et al., 1996; Chapter 3), while high levels of the conjugated forms are a sensitive indicator of their presence. In addition, elimination of the conjugated steroids is primarily by excretion in the urine or feces, compared to passive elimination of free steroids through the gills (Scott and Vermeirssen, 1994), so the retention of conjugated forms in the plasma

Table 10: Relationship between pheromone responsiveness and steroid hormone profiles in plasma of herring

Maturity class (sampling date)	Pheromone response class[2]	Steroid concentration (ng free steroid equivalent/ml; mean ± S.E.)[1]											n
		17α-P	3α,17-P-5β		17,20β-P			T		Keto-T		Cortisol	
		Free	Free	Gluc	Free	Sulph	Gluc	Free	Gluc	Free	Gluc	Free	
MALES													
Newly spermiating	+ve	114.61* ± 9.49	106.86* ± 21.35	155.67 ± 38.45	6.90 ± 1.43	6.25 ± 2.41	84.80 ± 16.55	3.73 ± 1.18	119.67 ± 22.40	0.82 ± 0.30	33.80 ± 6.10	41.58 ± 7.10	5
(Mar. 6, 1992)	-ve	25.30 ± 8.28	21.64 ± 6.37	163.28 ± 62.43	4.54 ± 1.52	3.45 ± 0.93	31.56 ± 15.74	4.84 ± 0.71	218.25 ± 41.22	2.27 ± 1.17	43.70 ± 7.38	42.06 ± 6.15	10
Spermiating & holding	+ve	7.44 ± 4.87	13.94 ± 8.16	17.80 ± 3.37	1.26 ± 0.69	0.22 ± 0.13	10.97 ± 5.02	1.05 ± 0.14	65.37 ± 29.40	< 0.2	12.76 ± 7.07	52.61 ± 19.07	3
(Apr. 21 & 22, 1994)	-ve	10.09 ± 3.49	18.15 ± 6.44	86.83 ± 31.98	1.35 ± 0.17	4.51 ± 2.62	27.96 ± 12.97	0.82 ± 0.06	61.87 ± 21.82	< 0.2	14.57 ± 5.47	153.92 ± 76.85	5
	Spont.	5.30 ± 1.72	5.82 ± 1.575	87.79 ± 39.07	1.11 ± 0.31	9.42 ± 4.55	24.03 ± 4.36	0.91 ± 0.06	48.05 ± 7.5	0.27 ± 0.06	21.61 ± 4.38	84.06 ± 3.27	4
FEMALES													
(Ovulated & holding	+ve	4.01 ± 0.68	5.77 ± 1.39	6.86 ± 1.58	1.34 ± 0.87	11.32 ± 6.42	3.41 ± 1.03	1.21 ± 0.45	5.88 ± 1.13	n/a	n/a	80.46 ± 5.10	6
Apr. 21 & 22, 1994)	-ve	8.01 ± 2.22	10.8 ± 3.37	21.00 ± 8.80	0.97 ± 0.25	4.68 ± 1.79	12.53 ± 2.80	0.94 ± 0.08	12.77 ± 3.25	n/a	n/a	89.90 ± 3.55	6

[1] Free=free steroid, Gluc=glucuronated steroid, Sulph=sulphated steroid

[2] +ve = responsive to pheromone, -ve -= non-responsive to pheromone stimulus, spont = spontaneous spawning without overt pheromone stimulus.

* significantly different from levels in non-responsive fish (Bonferronig-corrected α=0.005 for males, 0.006 for females)

may be more prolonged than that of the parent free forms. Plasma levels of conjugated steroids thus have been used in fish as an indicator of the presence of key biologically active steroids that are present only in a transitory fashion, are produced only in small quantities, or are metabolized very rapidly (Scott and Vermeirssen, 1994). The highly variable levels of glucuronated 3α,17-P-5β in all groups and the anomalously elevated levels in non-responsive newly ripe male fish (relative to levels of the free steroid) thus may indicate that free 3α,17-P-5β was also recently elevated prior to sampling and possibly produced in a pulsatile fashion. However, other explanations are also possible: for example, conjugation or elimination rates could be different in responsive and non-responsive fish due to increased competition for the glucuronating enzyme by 17,20ß-P (Carolsfeld et al., 1996; Chapter 3).

The role of 3α,17-P-5β in the reproduction of herring is still unknown. Stacey et al. (1987) indicate that 5β-reduction is a common characteristic of fish pheromones. Likewise, Canario and Scott (1990) suggest that 5β-reduced pregnanes are potentially a rich source of diverse pheromones produced coincidentally with reproductive events that are sensitive to pheromones. As an example, Schoonen and Lambert (1986) found that in the male African catfish synthesis of 3α,17-P-5β and related steroids was higher in wild spawning fish than in non-spawning captive fish, and Resink et al. (1989) found that 3α,17-P-5β is a male pheromone used to attract females in this species. Thus, coincident pheromonal responsiveness in herring and high levels of 3α,17-P-5β, and 17-P suggest a pheromonal role for these or related steroids. However, I was unable to detect a response to 3α,17-P-5β or its glucuronide in the herring spawning bioassay (Chapter 3), though the sulphated form has not yet been tested. Sherwood et al. (1991) found earlier that 17-P is

inactive in the herring spawning assay. However, I have shown recently that the spawning pheromone of herring consists of at least two components that act synergistically, one being 17,20ß-P sulphate (Chapter 5). Thus, 17-P or 3α,17-P-5ß and its conjugates may also be part of a synergistic pheromonal mixture and/or the high plasma levels of the steroids may be related to the production of the actual pheromone components.

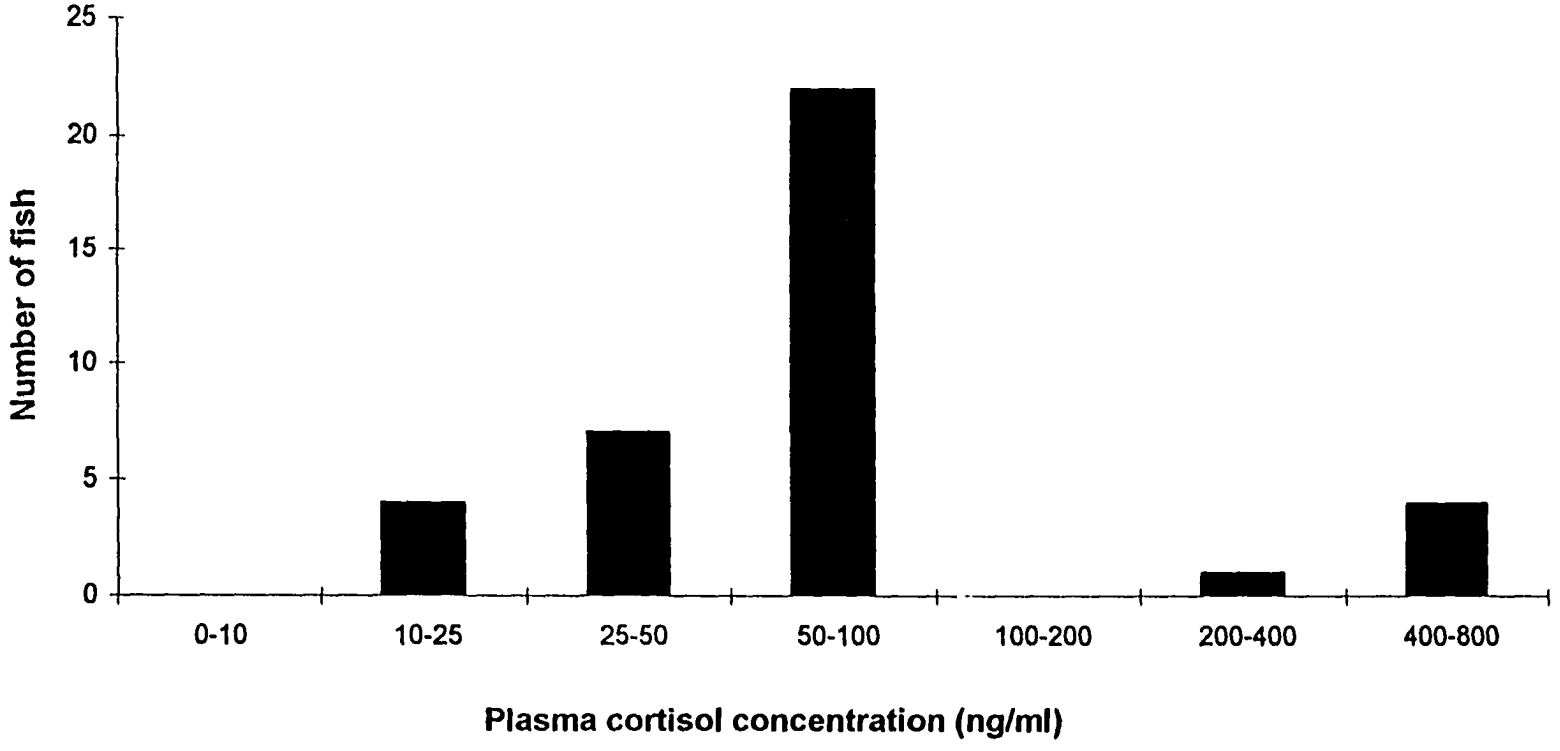
The apparently lower level of glucuronated 17,20ß-P in the plasma of responsive ripe-and-holding female fish, compared to non-responsive fish of this class, may be biologically significant, even if not statistically significant after a Bertollini correction for comparisons of a number of factors to distinguish these groups, as discussed above for differences in milt volume. If present, this difference between female fish that are responsive or non-responsive to the pheromone suggests that the responsive fish are more advanced in reproductive maturity, because steroid levels are dropping in the fish as the season progresses (Carolsfeld et al., 1996; Chapter 3). Also, the responsive fish may have spawned to some extent, as suggested by the lower gonadosomatic index compared to the non-responsive fish (Table 9). It is unclear if this indicates that partial spawning of female herring is a regular occurance or perhaps an artifact of captivity.

I was unable to detect differences between plasma steroid levels or morphological characters of responsive, non-responsive, and spontaneous ripe-and-holding male fish. Our sample sizes may not have been adequate to distinguish differences associated with responsiveness of fish in this group, and/or this group may be more diverse in maturity relative to responsiveness than the other groups. In particular, I found that "spontaneous" fish are likely to include both responsive and non-

responsive fish (Chapter 2) and appear to have the most variable gonadosomatic indices of male fish sampled at this date (Table 9). However, it is also likely that other factors beyond sexual maturity are influencing pheromone responsiveness, as indicated by our behavioral experiments (Chapter 2).

I was also unable to find differences in plasma levels of cortisol associated with responsiveness to the spawning pheromone, but the range of values that I determined varied greatly, from 14 to > 400 ng/ml, with a bimodal distribution (Fig. 17). Cortisol levels of unstressed herring are unknown, but the levels that I measured in most cases are elevated relative to the normal range of 12 ng/ml in unstressed trout (Goede and Barton, 1990) and 20 ng/ml in mullet (Thomas, 1990), but considerably lower than the 840 ng/ml reported by Scott et al. (1991) for herring. Elevated cortisol levels are a sensitive indicator of stressful events within 6-12 hours, but are not generally maintained at peak levels during chronic stress (Thomas, 1990). Thus, the extremely elevated levels that Scott et al. (1991) found and that I observed in some individuals (one mode of the histogram in Fig. 17), are likely to have been due to an unspecified stress during or just prior to sampling. The levels of cortisol I observed in the majority of fish indicate a mixture of fish that are either in a compensatory state for chronic stress of the captive situation or are recovering from stressful events that occurred some time before. Cortisol levels are believed to be proportional to the level of stress when it is applied, so the lack of a correlation between cortisol levels and responsiveness to the pheromone in the fish I used suggests that responsiveness is not directly related to acute stressful events. However, effects of stress in fish are numerous and generally difficult to assess (Sumpter et al., 1994), so

Fig. 17. Frequency histogram of concentrations of cortisol encountered in the plasma of herring in the present study. The observed range of measurements was 14.5 - 456 ng/ml.


25
20
15
10
5
0
Number of fish
0-10
10-25
25-50
50-100
100-200
200-400
400-800
Plasma cortisol concentration (ng/ml)

responsiveness to the pheromone in the herring may be related to stress in other fashions.

In conclusion, I have found evidence that responsiveness to the spawning pheromone is related to subtle differences in sexual maturity, but is unlikely to be related directly to stress.

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CHAPTER 5. IDENTIFICATION OF A COMPONENT OF THE SPAWNING PHEROMONE OF THE PACIFIC HERRING BY LIQUID CHROMATOGRAPHY / MASS SPECTROMETRY (LC/MS)[4]

ABSTRACT

Pacific herring have a pheromone in the milt (semen) and ripe testes that induces spawning in conspecifics of both sexes. Using liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS), I confirmed the identity of one of the active components of the pheromone as 17α,20ß-dihydroxy-4-pregnen-3-one sulphate (17,20ß-P sulphate), and isolated a proposed second component that is characterized by a negative ion of m/z 374. Neither of these components alone is active as a pheromone, and 17,20ß-P sulphate is present in immature testes as well, even though the latter do not contain pheromonal activity. I also confirmed the identity of two further primary compounds in purified testes extracts as cortisol sulphate and taurocholic acid, using LC/ESI-MS and multiple reaction monitoring (MRM) of LC/ESI-MS/MS tandem mass spectrometry.

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[4] A version of this chapter was submitted for publication with co-authors Dr. Michael Ikonomou (Institute of Ocean Sciences, Department of Fisheries and Oceans, Sidney, B.C.), Dr. Stephen Pleasance (Institute for Marine Biosciences, National Research Council, Halifax, N.S and Glaxo-Wellcome Laboratories, Beckenham, Kent, BR3 3BS, UK), Pearl Blay (Institute for Marine Biosciences, National Research Council, Halifax, N.S), and Dr. N.M. Sherwood (Biology Dept., University of Victoria, Victoria, B.C.). The experimental work was carried out with significant conceptual input from Drs. Ikonomou and Pleasance, as well as of other people as outlined in the acknowledgements; Ms. Blay and others provided significant technical assistance; Dr. Ikonomou also provided considerable interpretative input.

INTRODUCTION

Pheromones are chemical signals transmitted between individuals of a species for a variety of purposes (Karlson and Lüscher, 1959). Pheromonal influences on reproduction include the synchronization of maturation by priming pheromones and the triggering of reproductive behaviors by releasing pheromones (Wilson, 1963; Chapter 1). Identification of pheromones has relied on the development of suitable bioassays and the availability of appropriate analytical technology. For example, reproductive releasing pheromones of terrestrial insects are presently the best known of pheromones (Arn et al., 1992), largely because sensitive and relatively simple bioassays such as those based on electrophysiological recordings from antennae (electroantennograms) are available (Blum, 1985). The volatile components of these pheromones lend themselves well to analysis by gas chromatography (GC), and thus also to GC linked with electroantennograms (Struble and Arn, 1984) and GC linked with mass spectrometry (GC/MS).

GC/MS has also been the method of choice for identification of pheromones from other animals (Novotny et al., 1990), possibly due to the ready availability of instrumentation, high resolution of the technique, and large databanks of characterized spectra. However, in the case of less volatile compounds, such as those found in aquatic environments, the derivatization required for analysis generally destroys bioactivity. GC/MS spectra of a complex mixture of derivatized compounds also can be difficult to analyze, because the original molecules are extensively fragmented. Electrospray ionization (ESI) is an alternative, relatively non-destructive ionization technique for mass spectrometry that produces abundant ions of intact polar and thermally labile molecules. The process

is based on dispersion of the analyte-containing solution into droplets in an electrical field at atmospheric pressure. These droplets evaporate, resulting in the gentle transfer of intact ions from the solution to a gas phase for MS analysis (Covey, 1996). Solutions in a wide variety of commonly used solvents are suitable for analysis, making ESI ideal for coupling liquid chromatography (LC) with MS (Gilbert et al., 1996; Whitehouse et al., 1985). The LC/ESI-MS technique has been widely applied to the analysis of large biomolecules, but, although less commonly applied, it is also well suited to the analysis of small polar and thermally labile molecules in complex mixtures of compounds that are difficult to analyze with GC/MS. LC/ESI-MS becomes particularly powerful for this application when used in conjunction with tandem mass spectrometry (MS/MS). In MS/MS, parent ions from the initial ionization process are introduced into a reaction chamber where collision induced dissociation produces distinctive daughter ions that are analyzed by a second mass spectrometer. This process works best with a soft ionization process such as ESI, in that a compound is present in the first mass spectrometer primarily as a single molecular ion. Particular parent and daughter ions can be selected for analysis in MS/MS (select reaction monitoring, SRM), eliminating background noise and increasing sensitivity and specificity. When combined with the flexibility and resolving powers of modern liquid chromatography, LC/ESI-MS/MS is a powerful tool for confirmation of the identity of underivatized trace amounts of compounds in complex mixtures (Gilbert et al., 1996; Johnson and Yost, 1985). Thus, LC/ESI-MS techniques should be particularly suited for analysis of non-volatile pheromones such as those that are transmitted in an aquatic, rather than an aerial environment.

Aquatic reproductive pheromones of fish (Van Weerd and Richter, 1991) are among the few vertebrate pheromones known. These pheromones are of particular interest in that they appear to consist primarily of excreted hormones or hormone metabolites (Stacey and Sorensen, 1991), as predicted for aquatic pheromones by Kittredge and Takahashi (1972). In the goldfish, which has been proposed as a model in which to study fish pheromones (Stacey and Sorensen, 1991), free and conjugated reproductive steroids are released by the female during final reproductive maturation and then act as pheromones that induce increased spermiation in male goldfish (Dulka et al., 1987). F prostaglandins are involved in ovulation of the goldfish, and metabolites of F prostaglandins have been proposed to act as pheromones that induce spawning behavior of males (Sorensen et al., 1988; Sorensen et al., 1995b).

Olfactory sensitivity to reproductive pheromones appears to be enhanced in fish, relative to other compounds (Sorensen et al., 1987). Hence, electrophysiological recordings from the olfactory epithelium have been used to screen synthetic reproductive hormones and their metabolites for possible pheromonal activity (Sorensen and Scott, 1994). However, analysis of mixtures of unidentified compounds with olfactory assays is not yet possible in fish, and GC/MS analyses of pheromones in bioactive urine extracts have been carried out with some, but limited, success in identification (Resink et al., 1989). LC/ESI-MS techniques are logical alternative procedures to use in the purification, characterization, and identification of small, non-volatile compounds such as these, but to date have not been used for fish pheromones other than in our preliminary work (Carolsfeld et al., 1991).

The Pacific herring produces a pheromone in the milt (semen) that elicits spawning behavior in fully mature individuals of both sexes within three minutes (Stacey and Hourston, 1982). This pheromone may play a role in synchronizing the spawning of the herring, as these fish do not form pairs during the spawning of up to several million fish (Hay, 1985). The species does not lend itself well to olfactory recordings, so an alternative assay based on the behavioral response of individual fish in small aquaria was developed (Sherwood et al., 1991; Chapter 3). Using this assay the spawning pheromone was partially characterized, but its components could not be identified (Sherwood et al., 1991; Carolsfeld et al. 1992). In this chapter I report the further purification of the spawning pheromone and the development of LC/ESI-MS and LC/ESI-MS/MS methodology for the identification of three principal components of pheromonal extracts.

MATERIALS AND METHODS

My strategy for identifying the spawning pheromone of herring with LC/ESI-MS involved the following steps: 1) estimate molecular size of the spawning pheromone with techniques that are independent of mass spectrometry; 2) select the most likely pheromonal candidates from bioactive extracts of milt and testes purified with contrasting chromatographic techniques; 3) carry out tentative identification of the selected compounds; 4) confirm the identity of compounds in the extracts with LC/ESI-MS/MS and multiple reaction monitoring (MRM) experiments; and 5) test bioactivity of identified compounds, individually and in combination. As this initial approach was not sufficient to identify the spawning pheromone, I proceeded to develop a more extensive

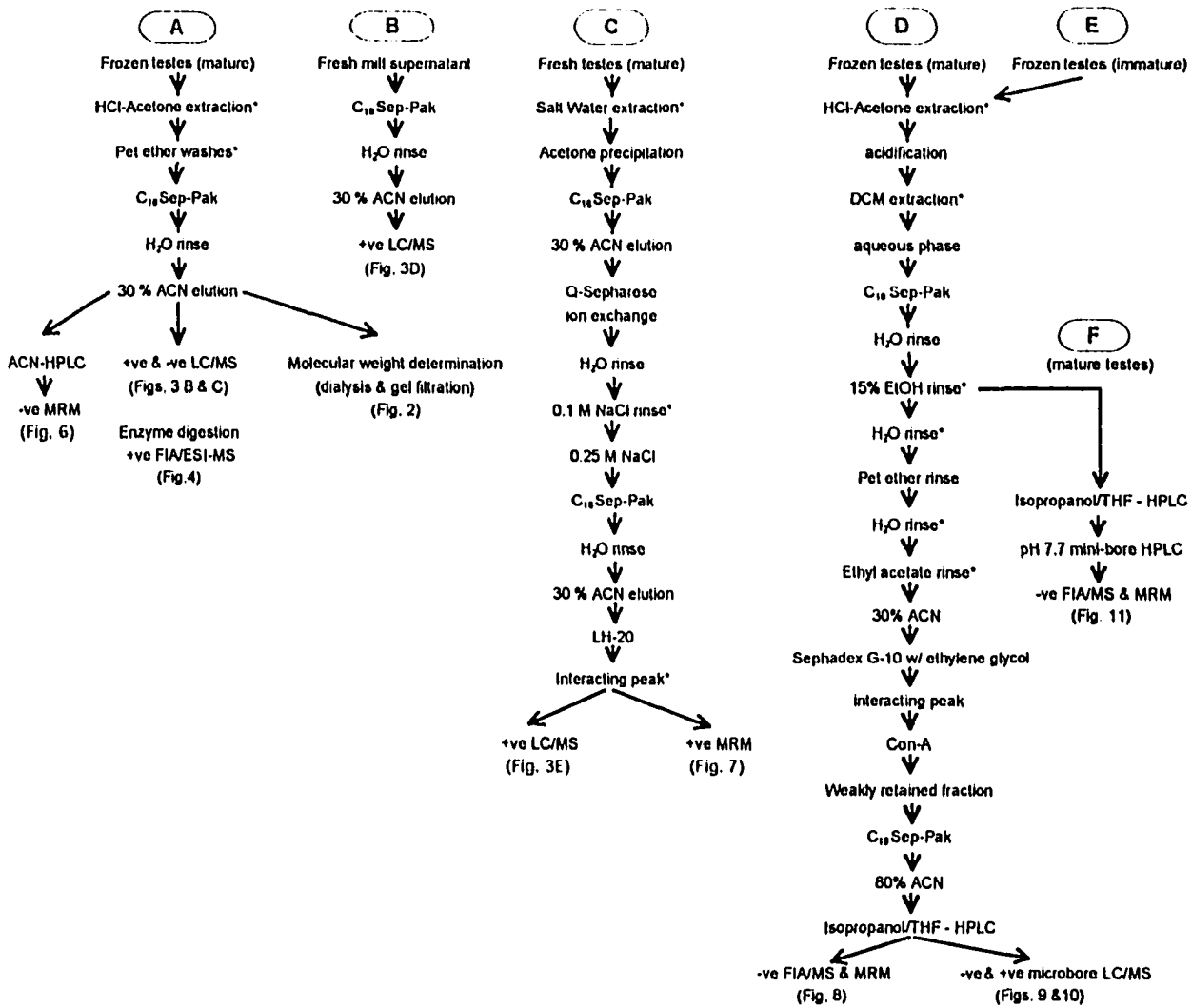
purification procedure, again with contrasting chromatographic steps, and compared the components of the resulting extracts with those of a parallel purification of immature herring testes. Extracts of immature testes, collected shortly before milt production was predicted to start, do not contain pheromonal activity (Stacey and Hourston, 1982), so I reasoned that these would provide a useful control material for selecting pheromonal candidates. All purification steps were guided by the spawning bioassays described below, whereas composition of purified fractions was investigated with positive and negative ion ESI-MS (Fig. 18).

Pheromonal material and fish:

Herring, with the gonads in the final stages of growth, were seined in Trincomali Pass (B.C. Canada) and transported by live barge to the Pacific Biological Station in Nanaimo, B.C., where they were maintained in net pens until spawning 2-3 months later. Immature testes (which do not contain pheromone) were collected from these fish within one week of capture, whereas mature (ripe) testes were collected from milt-producing fish approximately three weeks after capture. Milt was stripped from the ripe fish by gentle abdominal pressure before removal of testes. Testes and milt were either used fresh (protocols B and C) or frozen on dry ice and stored frozen at -20 °C for up to one year.

Herring for use in the bioassay were transferred from net pens to tanks with running natural sea water at the Pacific Biological Station. Individual ripe fish, characterized by easily extruded gametes (Hay, 1985), were selected from these tanks and were transported to the laboratory for use in bioassays.

Fig. 18. Outline of purification protocols used in the isolation of the herring spawning pheromone. See text for experimental details. Entries with an asterisk (*) indicate purification steps in which not all bioactivity is carried forward, whereas figure numbers indicate figures in which results of a purification procedure are presented. Abbreviations used: ACN, acetonitrile; DCM, dichloromethane; FIA, flow injection analysis; HPLC, high pressure liquid chromatography; LC-MS, linked liquid chromatography-electrospray mass spectrometry; LH-20, LH-20 Sephadex gel; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; THF, tetrahydrofuran; +ve, positive ionization; -ve, negative ionization.


A
Frozen testes (mature)
HCl-Acetone extraction*
Pet ether washes*
C_{18}Sep-Pak
H_2O rinse
30 % ACN elution
ACN-HPLC
-ve MRM
(Fig. 6)
+ve & -ve LC/MS
(Figs. 3 B & C)
Enzyme digestion
+ve FIA/ESI-MS
(Fig.4)
Molecular weight determination
(dialysis & gel filtration)
(Fig. 2)
B
Fresh mill supernatant
C_{18}Sep-Pak
H_2O rinse
30 % ACN elution
+ve LC/MS
(Fig. 3D)
C
Fresh testes (mature)
Salt Water extraction*
Acetone precipitation
C_{18}Sep-Pak
30 % ACN elution
Q-Sepharose
ion exchange
H_2O rinse
0.1 M NaCl rinse*
0.25 M NaCl
C_{18}Sep-Pak
H_2O rinse
30 % ACN elution
LH-20
Interacting peak*
+ve LC/MS
(Fig. 3E)
+ve MRM
(Fig. 7)
D
Frozen testes (mature)
HCl-Acetone extraction*
acidification
DCM extraction*
aqueous phase
C_{18} Sep-Pak
H_2O rinse
15% EtOH rinse*
H_2O rinse*
Pet ether rinse
H_2O rinse*
Ethyl acetate rinse*
30% ACN
Sephadex G-10 w/ ethylene glycol
Interacting peak
Con-A
Weakly retained fraction
C_{18}Sep-Pak
80% ACN
Isopropanol/THF - HPLC
-ve FIA/MS & MRM
(Fig. 8)
-ve & +ve microbore LC/MS
(Figs. 9 &10)
E
Frozen testes (immature)
F
(mature testes)
Isopropanol/THF - HPLC
pH 7.7 mini-bore HPLC
-ve FIA/MS & MRM
(Fig. 11)

Bioassay:

Extracts and purified fractions were assayed for pheromonal activity with individual herring in aquaria (Sherwood et al., 1991; Carolsfeld et al., submitted, Chapter 2). Briefly, male herring were screened for responsiveness to a 1ml stimulus of pheromone-containing milt (diluted 50% with sea water) in groups of five fish in 30 litre aquaria. Responsive fish were transferred to individual 66 litre aquaria and, after a 20 min recovery period, were exposed to 0.25 ml of the test solution pipetted onto the nares. A positive response to the substance, consisting of extension of the gonadal papilla and, at times, spawning, occurred within three minutes. Up to 12 further test substances were applied to the same fish at twenty minute intervals, followed by exposure to the milt stimulus used during the initial screening (1 ml of diluted milt) to confirm that the fish had retained responsiveness to the pheromone. Only fish that recovered fully from a response within twenty minutes were carried on for further testing. Up to seven such fish were tested in parallel, with the median response being used to assess the relative potency of the test solution. The concentration of an extract was calculated on the basis of the initial weight of tissue. Thus, a one-gram equivalent (g.e.)/ ml extract contained the equivalent of 1 gram of testes or milt per ml of extract.

Samples from extraction steps were tested individually after being diluted with distilled water. Fractions from the various chromatographic procedures described below were generally pooled in groups of 2 to 12 fractions for testing. These pools contained equal g.e. aliquots of each fraction. The chromatographic fractions were concentrated by vacuum centrifugation to remove organic solvents and made up to a constant volume with Milli-Q filtered water (Millipore) prior to testing.

Reagents:

All reagents used were analytical grade. Organic solvents and mobile phase modifiers were purchased from Aldrich (Milwaukee, WI) or BDH (Toronto, Canada), whereas water was freshly filtered and deionized with a Milli-Q filtration apparatus (Millipore, Bedford, MA). Synthetic taurocholic acid and cortisol sulphate were purchased from Sigma (St. Louis, MO), whereas sulphated 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) was synthesized from unconjugated 17,20ß-P by a process modified from Hadd and Blickenstoff (1969). This synthesis entailed reacting 10 mg of 17,20ß-P with 20 mg of sulfur trioxide-pyridine complex (Sigma) in 1 ml of pyridine overnight at 45 °C followed by one week at room temperature. Purification of the sulphated compound was carried out directly from the reaction mixture by high pressure liquid chromatography (HPLC) after removing the pyridine with vacuum centrifugation, reconstituting soluble components with water, and filtering with a 45 μm Millipore filter. The sample was separated on a Vydac 4.2 x 250 mm 201TP54 C_{18} column, and eluted with a linear gradient of 10-100% solvent B in 45 min at a flow rate of 0.5 ml/min delivered by a Varian 5000 HPLC. Solvent A for this procedure was 0.1% acetic acid, whereas solvent B consisted of 70% isopropanol, 5% tetrahydrofuran, and 25% aqueous (0.1%) acetic acid. The sulphated compound eluted as a single peak at about 36 minutes and was collected and reduced with vacuum centrifugation to remove organic solvents. Unreacted steroid eluted as a second peak at 41 minutes. The concentration of the sulphated compound was calculated by comparing the peak area of absorbance at 280 nm to a calibration curve of known concentrations of the free steroid eluted on the same program.

Mass Spectrometry:

Mass spectrometry was carried out either with a PE-SCIEX API-III mass spectrometer (Sciex, Thornhill, Ontario, Canada) linked to a Hewlett Packard HPLC pump or with a VG Quattro mass spectrometer (VG BioTech, Atrincham, UK) coupled with a Beckmann HPLC model 166 pump (Fullerton, CA). Both mass spectrometers are triple quatropole instruments equipped with an atmospheric pressure electrospray ionization source. The electrospray serves both as a liquid chromatography - mass spectrometry interface and as an ionization source for the mass spectrometer. Either positively or negatively charged ions are produced by this technique and are analyzed by the mass spectrometer in the corresponding mode. Compounds may ionize most readily in one or the other of these two ionization modes, depending on their chemical structure. Thus, I analyzed extracts in both ionization modes to ensure that I would miss as few components of the extracts as possible. The detection limit of the VG Quattro mass spectrometer for taurocholic acid was found to be 0.05 ng in the negative ionization mode and 0.15 μg in the positive ionization mode, as calculated from typical signal:noise ratios.

Flow injection analysis electrospray mass spectrometry (FIA/ESI-MS): FIA/ESI-MS was carried out by injecting 10 or 20 μl aliquots of samples directly into the flow of the carrier fluid (50% acetonitrile with 0.1% acetic acid) at 2-2.5 minute intervals, using a flow rate of the carrier of 20 μl/min. Blank injections of aliquots of the carrier fluid itself or of water were injected before and after analysis to check for system carry-over. Depending on the anaylsis, the MS was operated in the full scan mode covering the mass range m/z 150 to 1000 or in the select ion

monitoring (SIM) mode where particular molecular ions of the compounds of interest were monitored.

Liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS): Analysis of the initial purification processes was carried out on 20 μl samples with LC/ESI-MS using a Vydac 201TP54 C_{18} column with a linear gradient of 10-100% B in 45 minutes at a flow rate of 1 ml/min (solvent A: 0.1% acetic acid; solvent B: 0.1% acetic acid in acetonitrile). Post-column flow was split with 10-30 μl/min directed to the mass spectrometer; ions of m/z 200 to 800 were monitored, unless otherwise indicated. Preliminary analyses (not shown) monitored ions of m/z 150-1000 to determine which mass range contained significant signals. The more highly purified testes extracts were analyzed with LC/ESI-MS using a micro-bore C_{18} column, as described below.

Tandem mass spectrometry (MS/MS) analysis: IA/ESI-MS/MS) : Synthetic standards and an HPLC-purified component with m/z 516 (positive ion) that was found to be of interest in the initial purifications (see below) were subjected to FIA/EIS-MS/MS analysis. This was carried out by injecting aliquots of 20 μl of the sample into the flow of the carrier fluid (50% acetonitrile) as described for FIA/EIS-MS described above. The principal ion of interest was selected by the first mass spectrometer and directed into a collision chamber filled with argon gas. The principal ion undergoes collisionally-induced dissociation (CID) as it collides with the gas molecules and forms characteristic fragment ions depending on its chemical structure. The fragment ions are analyzed by the second mass spectrometer. Typical collisional energies used were 60 to 100V and the collision gas pressure was approximately 6×10^{-4} mBar. Fragment ions of

the mass range of m/z 50-600 were monitored for these analyses. Both positive and negative ion FIA/ESI-MS/MS was carried out on the synthetic standards, selecting for dissociation either the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions, respectively. Solutions with a concentration of 10^{-5} M of standards were used for negative ion FIA/MS/MS, whereas a concentration of 10^{-3} M was used for positive ion FIA/MS/MS of these compounds.

LC/ESI-MS/MS-MRM: Adequate HPLC separation of the three compounds was achieved with an analytical C_{18} column and the same program used for initial LC/ESI-MS described above (10-100% acetonitrile with 0.1% acetic acid in 45 minutes). The HPLC separation and the results from the FIA/ESI-MS/MS experiments on the synthetic compounds (see Results section) provided the basis for designing the proper positive and negative ionization MRM experiments for detecting the presence of 17,20ß-P sulphate, cortisol sulphate, and taurocholic acid in the semi-purified and purified testes extracts, as described below. Transitions of representative molecular ions to characteristic product ions of all three compounds were monitored together in negative ion LC/ESI-MS/MS with MRM of the semi-purified testes extract, whereas positive ion LC/ESI-MS/MS analysis of the ion exchange/LH-20 testes extract was carried out for only 17,20ß-P sulphate and cortisol sulphate. Transitions of the de-protonated molecular ion to product ions of m/z 97 and 80 were monitored for 17,20ß-P sulphate ($[M-H]^-$ m/z 411) and cortisol sulphate ($[M-H]^-$ m/z 441), whereas only the transition to the product ion of m/z 80 was monitored in the analysis for taurocholic acid ($[M-H]^-$ m/z 514). In the case of 17,20ß-P sulphate, the absence of a m/z 80 product ion despite the sulphate moeity was considered diagnostic. Positive ion transitions to

product ions that were monitored for analysis of 17,20ß-P sulphate ($[M+H]^+$, m/z 413) were m/z 110 and 333, whereas product ion m/z 122 and 363 were monitored for the analysis of cortisol sulphate ($[M+H]^+$, m/z 443).

Extraction and purification:

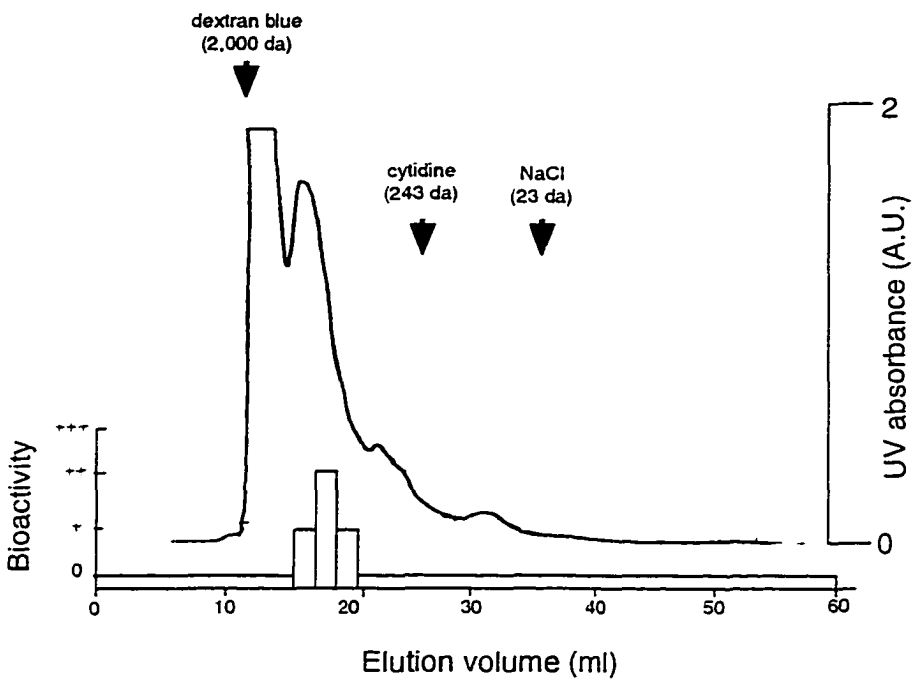
A) Molecular weight determination:

Molecular weight of the pheromone in "Sep-Pak-purified" testes extracts (Protocol A; Fig. 18) was estimated by gel filtration and dialysis (Fig. 19). For this purification, frozen testes were powdered in a blender with liquid nitrogen, then extracted for three hours in cold acetone (5ml acetone/g testes) acidified with 3ml 1N HCl/ 100ml acetone. The vacuum-filtered solids (Whatman no. 1 filter paper) were extracted again for 30 min with an acetone-0.01N HCl mixture (3:1, v/v) in a ratio of 1 ml acetone mixture/g testes. The filtrates were combined and washed with 5 aliquots of petroleum ether to remove non-polar lipids and acetone. Remaining acetone was removed from the aqueous phase with vacuum centrifugation, after which the extract was loaded onto columns of 6 primed C_{18} Sep-Pak cartridges (Waters, Milford, MA) in aliquots of up to 500 g.e. The column was rinsed with 20 ml of water, after which pheromonal material was eluted with 20 ml of 30% acetonitrile (ACN).

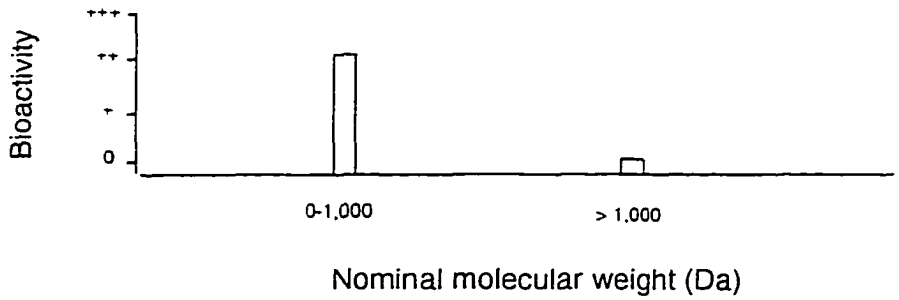
Gel filtration: A 0.65 ml sample of Sep-Pak-purified testes extract (40 g.e.) was applied to a 1 x 47 cm column of Sephadex G-10 and eluted with 20% ethanol at a gravitational flow rate of approximately 0.3 ml/min. Absorbance at 254 nm was monitored with a Gilson 112 detector, and fractions of 1.2 ml volume were collected. These were

Fig. 19. Determination of molecular weight of pheromone in Sep-Pak purified testes extract by A) gel filtration and B) dialysis. Gel filtration was carried out in a 1 x 47 cm column of Sephadex G-10 eluted with 20% ethanol at a gravitational flow rate of approximately 0.3 ml/min, monitoring UV absorbance at 254 nm. Dialysis was carried out for four hours with SpectraPor 9 dialysis tubing. Bioactivity is presented as a median response of 5-7 fish showing spawning (+++), full extension of the gonadal papilla (++), partial extension of the gonadal papilla (+), or no response (0).

A. Gel filtration



B. Dialysis



assayed in pools of 4 fractions at a concentration of 5 g.e./ml. Subsequently, pairs of fractions in the bioactive region were assayed at a concentration of 10 g.e./ml. A 350 µl cocktail of 100 µl of 4 mg/ml Dextran Blue (Pharmacia, Uppsala, Sweden; MW 2000), 25 µl of 20 mg/ml of cytidine (Sigma, MW 243.2), and 200 µl saturated NaCl were chromatographed on the same column to indicate distribution of molecular sieving.

Dialysis: 3 ml (900 g.e.) of Sep-Pak-purified testes extract was placed in 4 cm of presoaked SpectraPor 9 dialysis tubing (1000 Da nominal cut-off) and stirred in 300 ml of water for 3.5 hr at room temperature, followed by 30 min at 32°C. Contents of the beaker and remaining contents of the dialysis tubing were concentrated on columns of 3 C_{18} Sep-Paks, eluted with 80% ACN, and tested for bioactivity at a concentration of 300 g.e./ml (Fig. 19 B).

B) Selection of pheromonal candidates:

The initial selection of pheromonal candidates was based on LC/ESI-MS analysis of semi-purified milt and testes extracts, as well as of a further purified testes extract. Development of the purification protocols was guided by bioassays for pheromonal bioactivity at each step, as described above, while LC/ESI-MS was carried out as described in the mass spectrometry section above. Purification methods used for this work were (Fig. 18 A-C):

Semi-purified testes extract: Frozen testes were extracted and semi-purified on C_{18} Sep-Pak cartridges as for the molecular weight determination described above (Protocol A, Fig. 18). An 80 g.e. aliquot of this extract was chromatographed on a Beckman model 166 pump to provide fractions for bioassay. A Vydac 201TP54 C_{18} column was used

for separation with a linear gradient of 10-100% B in 45 minutes at a flow rate of 1 ml/min (solvent A: 0.1% acetic acid; solvent B: 0.1% acetic acid in acetonitrile). These fractions were assayed at a concentration of 20 g.e./ml in pools of 7 and 10 fractions to indicate the bioactive region, and then in pools of 3 fractions within the bioactive region.

Milt supernatant: Freshly collected milt was mixed with an equal volume of filtered sea water and centrifuged for 15 min at 5,000 g (Protocol B, Fig. 18). The supernatant, and two subsequent rinses of the centrifuged pellet, were combined for solid phase and HPLC purification as described for the testes extract, and assayed for bioactivity in an identical manner.

Ion exchange/LH-20-purified testes extracts: Approximately 3 kg of fresh intact testes were rinsed in flowing sea water and were then lanced with a scalpel and divided into 100 g aliquots (Fig. 18 C). Each aliquot was combined with an equal weight of filtered sea water and shaken vigorously prior to centrifugation at 5000 g for 15 min at 4°. The resulting pellets were rinsed twice with a further two aliquots of 50 ml of filtered sea water. The supernatants were combined (total volume 7.7 l) and large proteins were precipitated with the addition of 600 ml of cold acetone per liter aliquot of extract. The extract was stored in this state for about 1 month at 4°C, after which the acetone was removed with vacuum centrifugation. The resulting supernatant was loaded onto columns of 8 primed C_{18} Sep-Pak Plus cartridges in aliquots of 500 g.e. and eluted as described for the milt.

Acetonitrile was removed from the eluted fractions with vacuum centrifugation. The fractions were combined and applied to a Q-Sepharose (Pharmacia) ion exchange column (2.5 x 9.5 cm) in one aliquot of 615 g.e. and one of 2400 g.e. The samples were loaded onto

the column, which was then rinsed sequentially with 50 ml of water and 100 ml of 0.1M, 0.25M, and 1M NaCl, at a gravitational flow rate of approximately 5 ml/min. A single fraction was collected from each elution step with collection beginning at the time of application of each solvent. The bioactive 0.25 M fractions were pooled from the two runs, desalted on a column of 5 Sep-Pak cartridges eluted with 15ml of 30% ACN, and then chromatographed on a LH-20 column.

A 1 ml aliquot of the desalted ion exchange fractions (1200 g.e.) was chromatographed on a 45 x 1 cm LH-20 Sephadex (Pharmacia) column eluted with water at a flow rate of 0.5 ml/min (Fig. 18 C). The samples were spiked with 6 drops of saturated NaCl prior to chromatography to permit the visualization of the end of the molecular sieving range by noting changes in refractiveness. Ultraviolet (UV) absorbance was also monitored at 220 nm, and 3 fractions were collected corresponding with large molecules (11-26 ml), small molecules (27-31 ml), and interacting compounds (32-45 ml). Bioactivity was primarily found in the fraction of interacting compounds.

Selection of pheromonal candidates: LC/ESI-MS analysis was carried out on 20 µl aliquots of each of the semi-purified testes and milt extracts and the ion exchange/LH-20 purified testes extract described above. The analysis was carried out with the same HPLC column and program used to characterize the bioactivity in these extracts, with 30 µl of the post-column flow carried to the mass spectrometer. Analyses with both positive and negative ionization modes were carried out to allow detection of compounds that ionized in either mode. Pheromonal candidates were considered to be principal compounds that eluted within 12 and 16 min in the analysis of the extracts, comparable to the elution time of bioactivity in testes extract. A number of synthetic steroids and

prostaglandins considered to be likely candidates as pheromones in the literature were run previously with the same protocol (Carolsfeld et al., 1991). Three ions were considered to be of principal interest on the basis of predominance in at least one of the ionization modes and in two of the extracts examined with the LC/ESI-MS analysis (Table 11; Fig. 20). These were: positive ions m/z 413, 443, and 516, corresponding with negative ions 411, 441, and 514. Two further positive ions of m/z 181 and 241 were abundant in some crude testes and milt extracts, but were not investigated further.

C) Identification of pheromonal candidates:

The positive ions at m/z 413 and 443 correspond to expected protonated molecular ions of 17,20ß-P sulphate and cortisol sulphate, respectively. These compounds were previously reported from milt extracts using radioimmunoassay procedures (Scott et al., 1991), so identity of these ions in the extracts was investigated directly with LC/ESI-MS/MS using MRM experiments as described above.

The identity of the positive ion of m/z 516 was investigated by digestion with sulfatase and glucuronidase enzymes and with MS/MS characterization. The results from these experiments suggested that the compound may be taurocholic acid (see Results section), so its presence in the extracts was subsequently investigated with MRM analysis as for the other two compounds.

Enzyme digestion: An aliquot of the Sep-Pak-purified testes extract was subjected to enzymatic digestion with glucuronidases with and without sulfatase activity to test for conjugate groups on the positive m/z 516 ion. Four 0.1 ml aliquots of extract were dried in a vacuum centrifuge, reconstituted with 0.1 ml of water, and combined with one of:

Table 11: Principal ions of interest in electrospray-mass spectrometry of herring testes and milt extracts purified and analyzed with different techniques.

					Mass spectral analysis technique[1]			
					Sep-Pak Purified[2] Testes[2]		Sep-Pak Purified Milt	Ion-exch./LH-20 Testes
	Molecular ion [3]		MS/MS Fragment ions		A[2]	A	B	C
Compound	+ve[4]	-ve	+ve	-ve	+ve	-ve	+ve	+ve
17,20β-P sulphate	413	411	110,333	97	LC/ESI-MS	MRM	LC/ESI-MS	MRM
cortisol sulphate	443	441	122, 363	80, 97	-[5]	-	-	MRM
taurocholic acid	516	514	462, 480, 498	80	LC/ESI-MS	MRM	LC/ESI-MS	MRM
unknown	181	-	-	-	LC/ESI-MS	-	LC/ESI-MS	-
unknown	241	239	-	-	LC/ESI-MS	LC/ESI-MS	-	-
unknown	-	374	-	-	-	-	-	
unknown	-	443	-	-	-	-	-	-
unknown	-	496	-	-	-	-	-	-
unknown	-	498	-	-	-	-	-	-

[1] purification protocol and source material; letter corresponds to purification protocol outlined in Fig. 1

[2] mass spectrometric analysis procedure: LC/ESI-MS, liquid chromatography/elsctrospray-mass spectrometry; MRM, LC/ESI-MS/MS tandem mass spectrometry with multiple reaction monitoring; FIA/ESI-MS, flow injection analysis/electrospray mass spectrometry of HPLC fractions injected in sequence.

[3] molecular ions for known substances and observed ions of unknown compounds

[4] Ionization mode: positive (+ve) and negative (-ve)

[5] "-" indicates that no ion comparable to the one in this line of the table was observed in a particular purification

Table 11: Principal ions of interest in electrospray-mass spectrometry of herring testes and milt extracts purified and analyzed with different techniques (Cont'd).

			Mass spectral analysis technique[1]			
			Extended	Microbore HPLC: testes		Minibore HPLC
			Testes	mature	immature	testes
	Molecular Ion [3]		D	D	E	F
Compound	+ve[4]	-ve	-ve	-ve	-ve	-ve
17,20β-P sulphate	413	411	MRM	LC/ESI-MS	LC/ESI-MS	MRM
cortisol sulphate	443	441	MRM	-	-	-
taurocholic acid	516	514	MRM	-	-	-
unknown	181	-	-	-	-	-
unknown	241	239	-	-	-	-
unknown	-	374	LC/ESI-MS	LC/ESI-MS	-	FIA/ESI-MS[4]
unknown	-	443	-	LC/ESI-MS	LC/ESI-MS	FIA/ESI-MS
unknown	-	496	-	LC/ESI-MS	LC/ESI-MS	FIA/ESI-MS
unknown	-	498	-	LC/ESI-MS	LC/ESI-MS	FIA/ESI-MS

[1] purification protocol and source material; letter corresponds to purification protocol outlined in Fig. 1

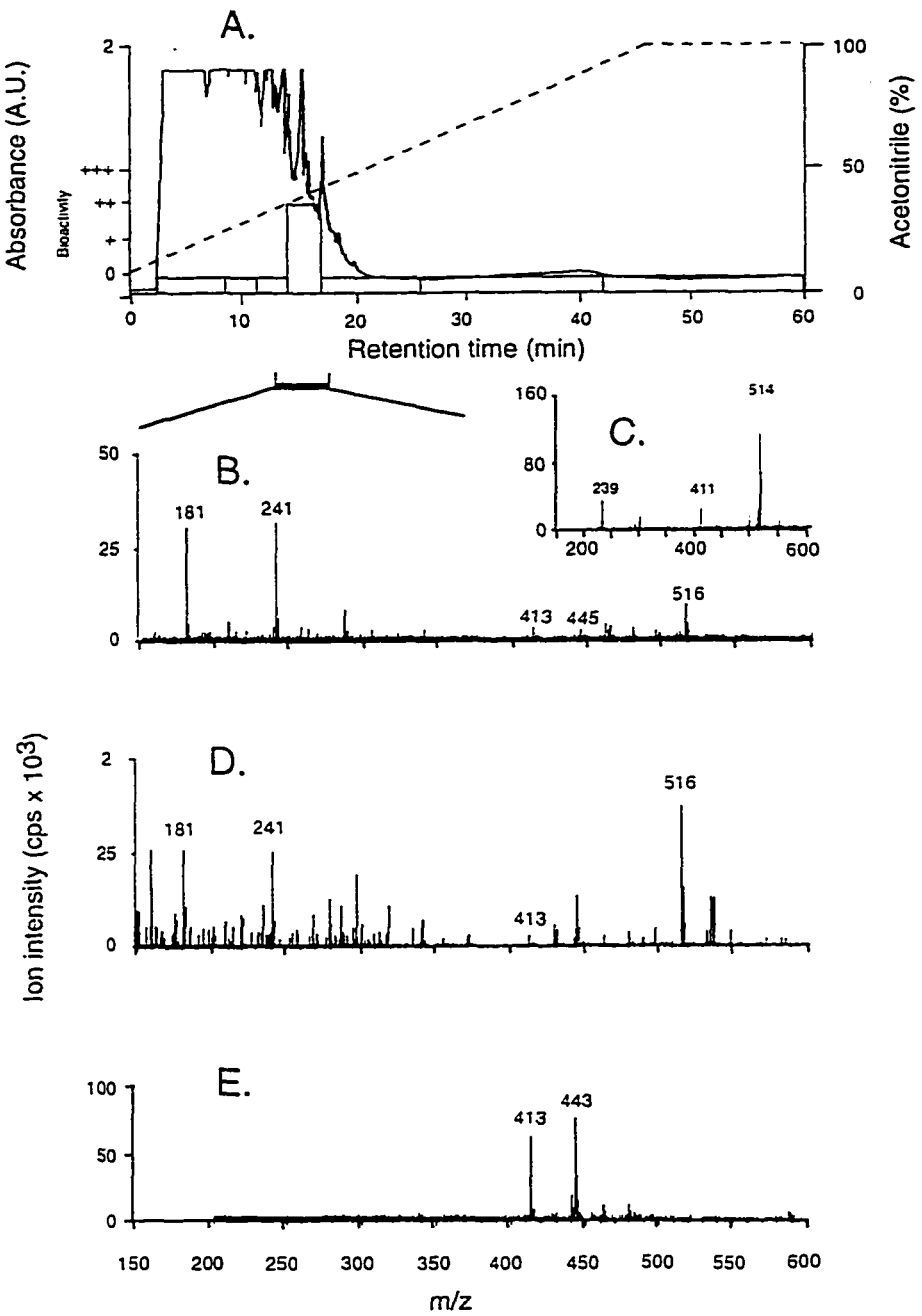
[2] mass spectrometric analysis procedure: LC/ESI-MS, liquid chromatography/elsctrospray-mass spectrometry; MRM, LC/ESI-MS/MS tandem mass spectrometry with multiple reaction monitoring; FIA/ESI-MS, flow injection analysis/electrospray mass spectrometry of HPLC fractions injected in sequence.

[3] molecular ions for known substances and observed ions of unknown compounds

[4] Ionization mode: positive (+ve) and negative (-ve)

[5] "-" indicates that no ion comparable to the one in this line of the table was observed in a particular purification

Fig. 20. A). Elution pattern of pheromonal bioactivity and UV absorption from HPLC separation of a Sep-Pak-purified testes extract, with bioactivity represented as in Fig. 19. B). Summed positive ion mass spectrum of bioactive region (12-16 min) of LC/ESI-MS separations of Sep-Pak purified testes extract with positive ionization. C). Same as B) but with negative ionization. D). Same as B), but of a Sep-Pak purified milt extract. E). Same as B), but of an ion exchange/LH-20-purified testes extract. Separation in all cases was achieved on a Vydac 218TP54 analytical C_{18} column with a linear gradient from 10% acetonitrile(ACN) / 0.1% acetic acid in water to 100% ACN/0.1% acetic acid in 45 min.


A.
Absorbance (A.U.)
Bioactivity
+++
++
+
0
2
100
50
0
Acetonitrile (%)
0
10
20
30
40
50
60
Retention time (min)
B.
50
25
0
181
241
413
445
516
C.
160
80
0
514
239
411
200
400
600
D.
Ion intensity (cps x 10^3)
2
25
0
181
241
413
516
E.
100
50
0
413
443
150
200
250
300
350
400
450
500
550
600
m/z

a) 0.1 ml water (control); b) 0.1 ml of a 0.1 mg/ml aqueous solution of ß-D-glucuronide glucuronosohydrolase (Type IX, Sigma No. G-3510); c) 0.1 ml of *Helix pomata* ß-glucuronidase with sulfatase activity (Type HP-2; Sigma No. G-7017, full strength); or d) 0.1 ml each of both of the two enzyme preparations. Aliquots of 0.1 ml of each enzyme with 0.1 ml of water were also incubated to test the molecular composition of the enzyme preparations. All mixtures were sonicated for 5 min, incubated at 37 °C for 2.5 hr, frozen overnight and subjected to FIA/MS of duplicate 10 µl aliquots of the samples as described in the mass spectrometry section above.

MS/MS analysis: Fractions of 1 min each were collected of the eluant of the LC/ESI-MS analysis of the semi-purifed testes extract that by-passed the mass spectrometer. Those fractions collected during the elution of the positive ion m/z 516 (fractions 17 and 18) were pooled and subjected to positive ion FIA/MS/MS analysis of this ion as described for synthetic standards above, except with the PE-SCIEX instrument rather than the VG Quattro instrument.

D) Bioactivity of initial pheromonal candidates:

The pheromonal candidates identified with the initial work described above were tested with the bioassay using a solution containing 10^{-3} M taurocholic acid, cortisol sulphate, and 17,20ß-P sulphate.

E) Extended purification of mature testes extract:

Initial pheromonal candidates identified with the work described so far proved to be devoid of pheromonal bioactivity. More extensive purification of testes extracts was thus considered necessary for identification of additional pheromonal candidates (Protocol D, Fig. 18).

Approximately 5 kg of frozen testes were powdered with liquid nitrogen and extracted in lots of 500g with acidified cold acetone as described for the semi-purified testes extract (Protocol A, Fig. 18). However, the filtrates in this protocol were not rinsed with petroleum ether, but rather were reduced directly with vacuum centrifugation. The resulting concentrate was acidified to pH 0.5 with 6N HCl and washed twice with dichloromethane (DCM; 50 ml/l of concentrate). The pH of the aqueous layer was corrected to 3 with 10 N NaOH, prior to loading each batch on a column of 6 primed C_{18} Sep-Pak Plus cartridges with a peristaltic pump. The extract was then eluted with a protocol designed for purification of prostaglandins, modified from Powell (1987). This consisted of elution sequentially with 20 ml each of: water, 15% EtOH, water, petroleum ether, water, ethyl acetate, 30% acetonitrile, and 80% acetonitrile. Excess solvent was removed after each step with a stream of 10-15 ml of air. The 30% acetonitrile fraction contained the majority of the bioactivity and was used for further purification. The eluates from each extraction batch were reduced to about 6 ml each (500 g.e.) by vacuum centrifugation. This sample was loaded onto a 2.5 x 45 cm Sephadex G-10 (Pharmacia) column preconditioned with 2 or more column volumes of 10% ethylene glycol monoethyl ether (EGMEE) in 0.1 M NaCl. Elution proceeded under gravitational flow with 100 ml of the initial EGMEE mobile phase, followed by 100 ml of 20% EtOH. The flow rate with the EGMEE was about 3 ml/min, decreasing to 1 ml/min with 20% EtOH. Absorbance of the eluate was monitored at 254 nm, and 1 min fractions were collected after an initial 100 ml of eluate had passed through the column. Fractions 77 to 95 corresponded to a secondarily retained bioactive U.V. peak and were pooled, reduced to 5 ml by vacuum centrifugation, and purified further with Concanavalin A (Sigma).

The pooled G-10 fractions of each batch were combined with 0.5 ml of sample buffer (50 mM sodium acetate and 10 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$). This sample was loaded onto a 1.8 x 11 cm Concanavalin-A column preconditioned with loading buffer (5 mM sodium acetate, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ in 0.1 M NaCl). The column was then rinsed with a further 40 ml of loading buffer, after which the pheromonal components were eluted with 40 ml of 0.01 M α-methyl D-glucoside. The column was used again after cleaning sequentially with 40ml 0.1M Tris HCl in 0.5M NaCl and 40ml 0.1M sodium acetate. Sugars were removed from the bioactive fraction by concentration on a column of three C_{18} Sep-Pak cartridges eluted with 10 ml of 80% acetonitrile. This eluate was reduced by vacuum centrifugation. The fractions of ten such Concanavalin-A runs were pooled, and one half of this pool (2,700 g.e.) was carried on to HPLC.

HPLC was carried out with a Beckman model 166 pump using a Vydac 215TP54 analytical C_{18} column. The sample (1.5 ml) was loaded in three injections of 500 μl each using a 1 ml injection loop, while keeping the column at initial conditions (1 ml/min of 10% solvent B). Elution was initiated 10 minutes after the initial injection with a gradient of 10 to 100% B in 40 minutes (solvent A: 0.1% acetic acid; solvent B: 70% 2-propanol, 5% freshly distilled tetrahydrofuran, 25% aqueous (0.1%) acetic acid), using a flow rate of 0.5 ml/min. UV absorbance was monitored at 220 nm, and 1 min fractions were collected. These fractions, once reduced to remove organic solvent, were bioassayed at 100 g.e./ml in pools of 30 fractions at either end of the run, and in pools of 5 fractions in the area of fractions 31-46. Subsequently, fractions in the bioactive region were bioassayed individually and in pools of three fractions at a concentration of 400 g.e./ml. Aliquots of fractions 31-45 and a pool of equal quantities

of fractions 38-41 were analyzed with negative ion FIA-ESI/MS to investigate molecular composition, as described above. The same pool of fractions 38-41 was also analyzed with negative ion LC/ESI-MS/MS and MRM for cortisol sulphate, 17,20ß-P sulphate, and taurocholic acid as described above.

Subsequently, 10 µl aliquots of fraction 40 (30 g.e.) were analyzed further with negative and positive ion C_{18} microbore LC/ESI-MS. This was carried out with a Vydac 218TP51 column coupled directly to the mass spectrometer and eluted isocratically with a flow of 30 µl/min of 25% acetonitrile containing 0.1% acetic acid.

F) Immature testes:

For comparison of results of protocol D to similar tissue without pheromonal activity, 1 kg of immature testes were collected 1 week prior to predicted initiation of milt production and were prepared and analyzed in the same manner as the mature testes of protocol D. Testes were previously shown to to be devoid of pheromonal activity until fully mature, as indicated by the presence of readily expressed milt (Sherwood et al., 1991; Stacey and Hourston, 1982). Only negative ion microbore LC/ESI-MS was carried out on the extract of this tissue, using a 10 µl aliquot of 20 g.e.

G) High pH mini-bore purified testes extract:

A compound of m/z 374 was observed in the negative ion mass spectrum of microbore HPLC-purified extracts of mature testes, but was absent in immature testes (see Results section). Negative ion FIA/ESI-MS of the Sep-Pak eluate fractions of protocol D indicated that the bioactive 15% EtOH fractions contained the highest concentrations of this

m/z 374 putative pheromone component, as well as a m/z 411 component (hypothesized to be 17,20ß-P sulphate; results not shown). An alternate purification scheme was thus designed using this Sep-Pak fraction and a high pH HPLC mobile phase that separated the components that resulted in the m/z 374 and 411 negative ions (Protocol F, Fig. 18). Frozen testes (approximately 1 kg) were purified as described for protocol D up to the point of Sep-Pak elution. The 15% EtOH Sep-Pak fractions (rather than the 30% ACN fractions) were carried further for purification. The fractions were reduced by vacuum centrifugation and chromatographed with three runs of the isopropanol HPLC program described in protocol D. Fractions 36-40 of these runs were pooled and reduced with vacuum centrifugation. Aliquots of 450 µl (560 g.e.) of this pool were applied to a Beckman 2.1 mm x 25 cm ODS mini-bore column while operating at initial conditions of 10% B (70% ACN, 30% solvent A; solvent A: 0.125% acetic acid corrected to pH 7.7 with concentrated ammonium hydroxide) pumped at a flow rate of 0.3 ml/min with a Beckman model 166 pump. A linear gradient to 30% B in 5 min was initiated 5 min after sample injection, followed by a shallower gradient to 60% B in 15 minutes. The program was completed with a steep gradient to 100% B in 5 minutes. U.V. absorbance at 220 nm was monitored and fractions of 0.3 ml were collected. Pools of 12 fractions were assayed at a concentration of 300 g.e./ml, whereas pools of four fractions within the bioactive zone were assayed at 1000 g.e./ml. Combinations of these smaller pools from a comparable HPLC run were assayed at 500 g.e./ml. The pool of fractions 18-21 was subsequently also tested in combination with synthetic 17,20ß-P sulphate (final concentration of 500 g.e./ml and 10^{-4} M, respectively). Molecular composition of the HPLC fractions in the bioactive region (fractions 16-41) was investigated with negative ion FIA/ESI-MS as

described above. Fraction 27, found to contain the majority of the m/z 411 ion, was then analyzed with negative ion LC/ESI-MS/MS and MRM for 17,20ß-P sulphate as described above.

RESULTS

My strategy for identifying the spawning pheromone with LC/ESI-MS techniques was to: A) determine the molecular weight of the pheromone with techniques independent of mass spectrometry, B) select likely pheromonal candidates by LC/ESI-MS analysis of semi-purified milt and testes extracts, C) deduce the identity of the pheromone candidates by various means, D) characterize the corresponding synthetic standards with LC/ESI-MS/MS, and confirm their identity in the extracts with LC/ESI-MS/MS and MRM, E) test the bioactivity of these initial pheromone candidates, F) purify extracts of both mature and immature testes more extensively and analyze these with LC/ESI-MS to indicate additional pheromonal candidates, and G) evaluate bioactivity of the additional pheromonal candidates. The results of this approach are summarized in Table 11.

A) Molecular weight estimation:

Pheromonal activity eluted from Sephadex G-10 with a 20% EtOH mobile phase in about 15 ml, a position intermediate between Dextran Blue at the void volume of 12 ml and cytidine at 27 ml (molecular weight of 240 daltons) (Fig. 19 A). Salt (NaCl) eluted at the column volume of 36 ml. The nominal exclusion limit of this gel is 700 Da, so I estimated the molecular weight of pheromonal components to be 300-500 Da. Pheromonal activity was present in the < 1000 MW dialysis fraction, but was absent in the larger molecular weight fraction (Fig. 19 B), in confirmation of the results of the gel filtration experiment.

B) Selection of initial pheromonal candidates:

Bioactivity eluted from the HPLC purification of milt and testes in 3 fractions, 13-15 (Fig. 20 A). The positive ion mass spectra of the 12-16 min area of the comparable LC/ESI-MS analysis of milt and testes showed principal ions of m/z 181, 241 and 516 (Fig. 20 B, D), whereas the negative ion spectrum showed principal ions of m/z 239, 411, and 514 in the testes extract (Fig. 20 C). A positive ion of m/z 413 was also detectable in the milt and testes extracts, but with relatively low intensity (Fig. 20 B, D).

Purification of salt water-extracted fresh testes with ion exchange and LH-20 chromatography resulted in two principal positive ions in the bioactive area of LC/ESI-MS elution: one of m/z 413 and one of m/z 443 (Fig. 20 E). These ions corresponded to the predicted masses of sulphated 17,20ß-P and sulphated cortisol, respectively, as shown in the analysis of the synthetic standards (see below).

C) Identification of pheromonal candidates:

The undigested testes extract used for the enzyme studies contained a distinct m/z 516 positive ion, which was absent in both enzymatic preparations. Digestion with aryl sulfatase caused the disappearance of this ion, whereas digestion with the β-glucuronidase did not, indicating the presence of a sulphate group on the m/z 516 ion (Fig. 21 A-C). In addition, the uneven predicted molecular weight of 515 Da suggests the presence of an odd number of nitrogen atoms. These characteristics suggest a taurine conjugated compound, leading to the tentative identification of the compound as taurocholic acid: a taurine conjugated steroid ($C_{26}H_{44}NO_7S$; MW 515.6). Positive ion CID of the m/z

516 ion produced three major fragment ions at m/z 462, 480, and 498, all separated by 18 daltons (Fig. 21 D) corresponding to neutral losses of H_2O and suggesting the presence of three hydroxyl groups in the parent ion, as are present in the CID mass spectrum of synthetic taurocholic acid (Fig. 22). The m/z 337 ion (Fig. 21 D) may correspond to a C_{21} fragment of the molecule with the alkyl side chain cleaved off, $[C_{21}H_{36}O_3]^+$. This may not have occurred during CID of synthetic taurocholic acid because of slightly different CID conditions (analysis of the synthetic compound was carried out on the VG Quattro mass spectrometer, whereas the natural substance was analyzed on the PE-SCIEX mass spectrometer).

The positive ions of m/z 413 and 443 (Fig. 20) and negative ions of m/z 411 and 441 correspond to expected ions of 17,20ß-P sulphate ($C_{21}H_{32}O_6S$) and cortisol sulphate ($C_{21}H_{31}O_8S$), respectively (Fig. 22; Table 11). Both compounds have previously been reported to be present in herring milt based on evidence from radioimmunoassays (Scott et al., 1991), and were adopted as likely structures for the compounds observed with the ESI-MS, as described above.

D). Characteristic CID fragmentation of synthetic standards and MRM identification in extracts:

Negative ion FIA/ESI-MS/MS with CID of 17,20β-P sulphate, cortisol sulphate, and taurocholic acid all produced fragment ions of m/z 80 and/or 97, corresponding to negatively charged sulphate ions $[SO_3]^-$ and sulphate ions with an attached hydroxyl group $[HSO_4]^-$ (Table 11, Fig. 22). The relative abundance of the two sulphate ions appears to be diagnostic of each compound: taurocholic acid only produced the m/z 80 ion, 17,20β-P sulphate produced primarily the m/z 97 ion, and cortisol sulphate produced both ions. This characteristic appears related to the

Fig. 21. Characterization of positive ion m/z 516 in Sep-Pak-purified testes extract by enzyme digestion and collision-induced dissociation with FIA/ESI-MS/MS. A). An extracted positive ion chromatogram for m/z 516 of a FIA/ESI-MS run with sequential duplicate injections of (1) undigested control sample, (2) ß-glucuronidase enzyme preparation without sample, (3) aryl-sulfatase enzyme preparation without sample, (4) ß-glucuronidase digested sample, (5) sulfatase digested sample, and (6) combined enzyme digested sample. B). Positive ion mass spectra of the undigested sample and (C) of the sulfatase-digested sample. D). A positive product ion spectrum of collisionally induced dissociation of the m/z 516 ion in fractions 17 and 18 of an HPLC purification of testes extract as described in Fig. 20.

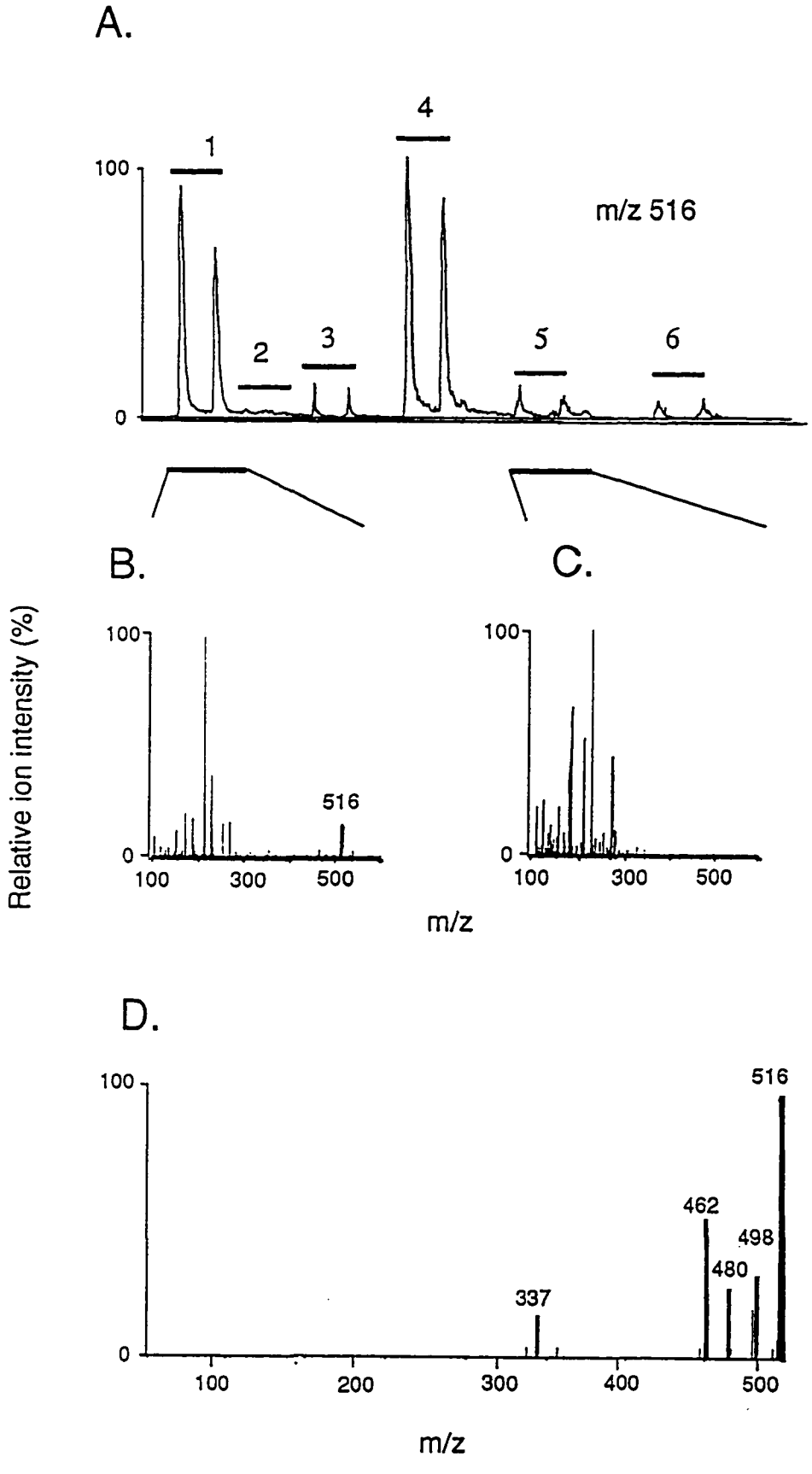
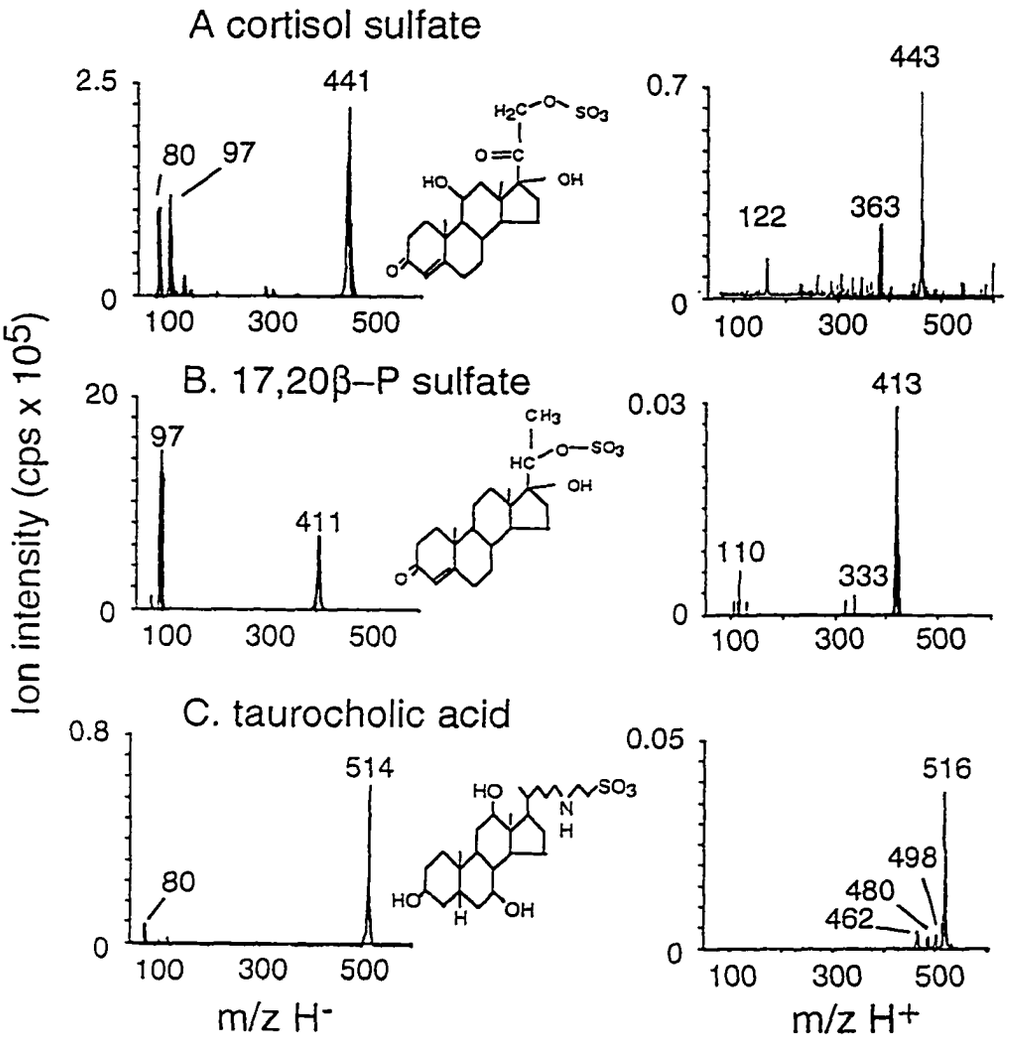

A.
1
2
3
4
5
6
100
0
m/z 516
B.
C.
100
0
516
100
300
500
Relative ion intensity (%)
m/z
D.
100
0
516
462
498
480
337
100
200
300
400
500
m/z

Fig. 22. Negative (left) and positive (right) product ion spectra of collisionally-induced dissociation FIA/ESI-MS/MS of synthetic standards of (A) cortisol sulphate, (B) 17,20ß-P sulphate, and (C) taurocholic acid. The mobile phase for MS/MS experiments was 50% acetonitrile for negative ionization and 50% acetonitrile with 0.1% acetic acid for positive ionization. Solutions of 10^{-5} M were used for the negative ionization experiments, whereas solutions of 10^{-3} M were used for positive ionization.


A cortisol sulfate
2.5
441
80
97
0
100
300
500
0.7
443
122
363
0
100
300
500
B. 17,20β–P sulfate
20
97
411
0
100
300
500
0.03
413
110
333
0
100
300
500
C. taurocholic acid
0.8
514
80
0
100
300
500
m/z H⁻
0.05
516
498
480
462
0
100
300
500
m/z H⁺
Ion intensity (cps x 10⁵)

proximity of the sulphate group to a hydroxyl group on the parent ion. Positive ion FIA/ESI-MS/MS with CID of 17,20ß-P sulphate and cortisol sulphate (Fig. 22) was characterized by fragment ions corresponding to the parent steroid without the sulphate group [M^+ - SO_3, m/z 333 and 363] and ions of uncertain structure (m/z 122 and 110) that may result from the cleavage of one of the steroid rings.

Negative ion FIA/ESI-MS/MS with MRM for characteristic fragment ions of 17,20ß-P sulphate, cortisol sulphate, and taurocholic acid indicated the presence of these compounds in the bioactive pool of HPLC fractions of the Sep-Pak-purified testes extract (Fig. 23), though cortisol sulphate was only present in trace amounts. Positive ion LC/ESI-MS/MS with MRM for product ions of 17,20ß-P sulphate and cortisol sulphate also indicated the presence of these compounds in the LH-20-purified testes extract (Fig. 24).

E) Bioactivity of initial pheromone candidates:

The solution of 10^{-3} M taurocholic acid, cortisol sulphate, and 17,20ß-P sulphate, was inactive in the spawning assay (in 0 / 7 fish).

F) Highly purified testes extract:

More extensive purification of a mature testes extract (protocol D) resulted in a bioactive pool of HPLC fractions 38-40 containing primarily the m/z 411 negative ion (17,20β-P sulphate), with traces of other ions, including ones of m/z 514 (taurocholic acid), 443 (unknown), 441 (cortisol sulphate) and m/z 374 (unknown)(Fig. 25 A, B). Confirmation of the presence of 17,20β-P sulphate, cortisol sulphate, and taurocholic acid in this extract was obtained from negative ion LC/ESI-MS/MS with an MRM

Fig. 23. A negative ion LC/ESI-MS/MS with MRM of the pool of bioactive fractions 12-16 from the testes extract of Fig. 19. A). Reconstructed total ion chromatogram of ions monitored in B-D. Product ions of B). 17,20ß-P sulphate ($[M-H]^-$ m/z 411), C) cortisol sulphate ($[M-H]^-$ m/z 441), and D) taurocholic acid ($[M-H]^-$ m/z 514) were monitored. The HPLC program used for separation was as described in Fig. 20. Molecular ions and fragment ion of m/z 80 and 97 were monitored (only m/z 80 for taurocholic acid). Arrows indicate the retention times of authentic standards when chromatographed separately.

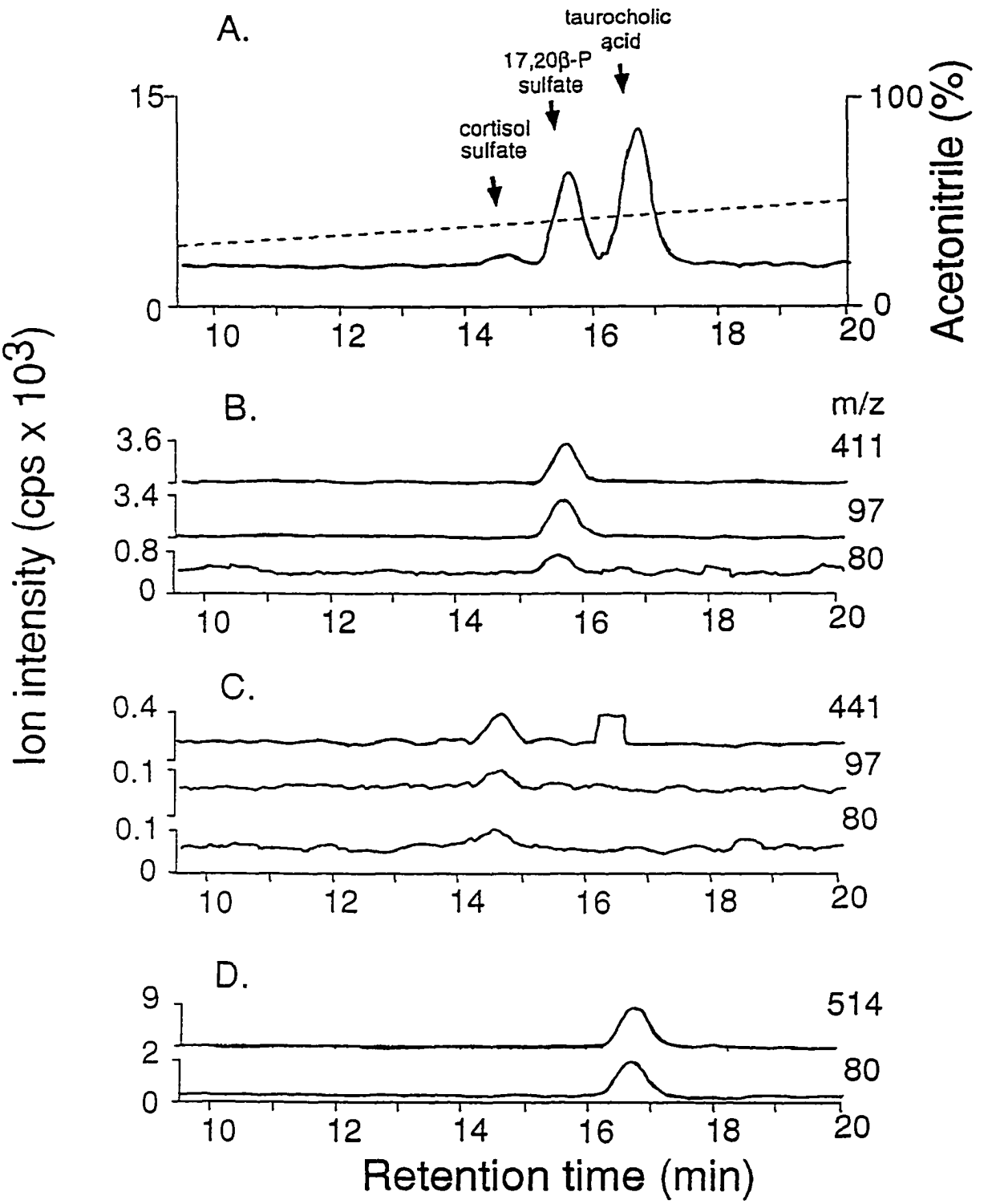
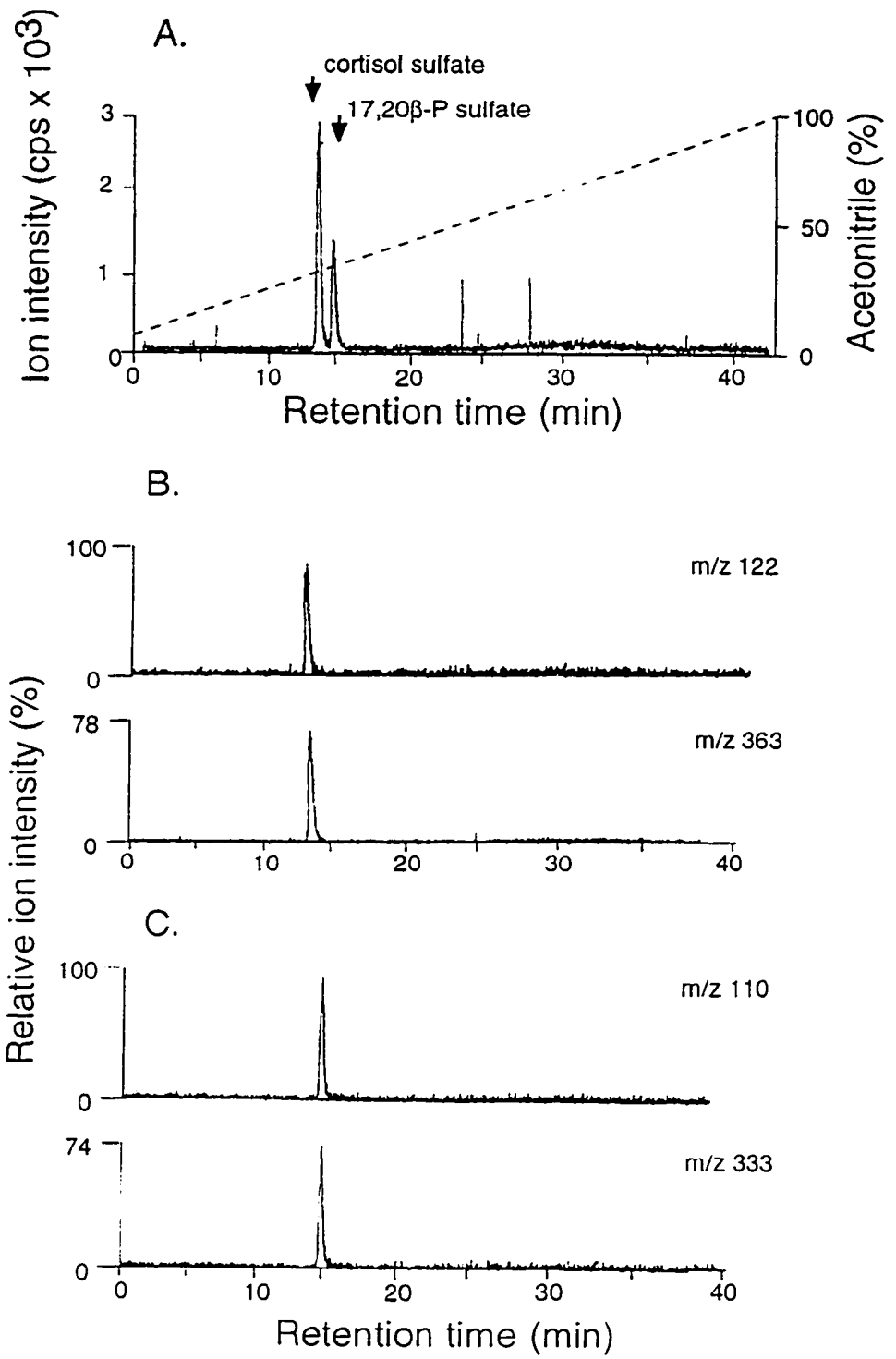

A.
taurocholic acid
17,20β-P sulfate
cortisol sulfate
15
0
100
0
Acetonitrile (%)
10
12
14
16
18
20
B.
m/z
3.6
3.4
0.8
0
411
97
80
C.
0.4
0.1
0.1
0
441
97
80
D.
9
2
0
514
80
Ion intensity (cps x 10³)
Retention time (min)

Fig. 24. A positive ion LC/ESI-MS experiment with MRM of a bioactive LH-20-purified testes extract (protocol C of Fig. 18). A). Reconstructed total ion chromatograph of ions monitored in B-C. Product ions of B) cortisol sulphate and C) 17,20ß-P sulphate were monitored. The HPLC program used is as described in Fig. 20, but analysis was carried out on a PE-SCIEX, rather than a VG Quattro, mass spectrometer. Fragment ions of m/z 122 and 363 were monitored to confirm the identity of cortisol sulphate, whereas fragment ions of m/z 110 and 333 were monitored to confirm the identity 17,20ß-P sulphate. Arrows indicate the retention times of authentic standards when chromatographed separately with this system.


A.
cortisol sulfate
17,20β-P sulfate
Ion intensity (cps x 10³)
Acetonitrile (%)
Retention time (min)
B.
m/z 122
m/z 363
C.
m/z 110
m/z 333
Relative ion intensity (%)
Retention time (min)

experiment, where the presence of characteristic fragment ions of these compounds was monitored (results not shown). Negative ion microbore LC/ESI-MS of fraction 40 of the bioactive zone resulted in two principal peaks of negative ions from the extracts of mature testes (Fig. 26) and immature testes (Fig. 10). The first of these peaks contained primarily a m/z 374 ion (unknown identity) in the mature testes extract (Fig. 26 B, C), but was slightly delayed and devoid of this ion in the immature testes extract (Fig. 27 B, C). The second peak consisted of m/z 411 (17,20β-P sulphate), 443 (unknown), and 496 (unknown) negative ions in both extracts (Figs. 26 B, C; 27 B, C). The expected positive ion m/z 413 was not found with positive ion microbore LC/ESI-MS of the same mature testes extract, but this is likely due to the lower sensitivity of the mass spectrometer to this compound in the positive ion mode.

Chromatography with high pH minibore C_{18} HPLC of the 15% EtOH eluate from the C_{18} Sep-Paks resulted in a separation of pheromonal components. A pool of fractions 18-26 was bioactive, but when subdivided into pools of three fractions each, the bioactivity was not found (Fig. 28 A). Negative ion FIA/ESI-MS of fractions 16-30 indicated the presence of the m/z 374 ion in fraction 20 and the 411 m/z negative ion in fraction 27 (Fig. 28 B). The other negative ions that eluted with m/z 411 in microbore HPLC (m/z 443, 496, and 498) eluted either with m/z 411 (m/z 496) or one (m/z 443) or two (m/z 498) fractions later from this mini bore program. Combination of the two 3-fraction pools containing these compounds succeeded in reconstituting the bioactive material (Fig. 28 C), suggesting the presence of two components of the pheromone that are individually inactive. Negative ion LC/ESI-MS/MS analysis with MRM for 17,20ß-P sulphate of fraction 27 confirmed the identity of the 411 m/z ion as this compound (not shown). Combination of synthetic sulphated

Fig. 25. A). Elution of UV absorbance and pheromonal bioactivity of mature testes from HPLC with an isopropanol mobile phase (Protocol D of Fig. 18). Separation on a Vydac 201TP54 C18 column was started after 10 minutes at initial conditions with a linear gradient of 10 to 100% solvent B in 40 min. Solvent A was 0.1% acetic acid, whereas solvent B consisted of 70% isopropanol, 5% tetrahydrofuran, 24.9% water, and 0.1% acetic acid, pumped at a flow rate of 0.5 ml/min. Bioactivity is presented as indicated in Fig. 20. B) Negative ion FIA/ESI-MS mass spectrum of a pool of fractions 38-42 of the HPLC separation shown in A), injected with 50% acetonitrile.

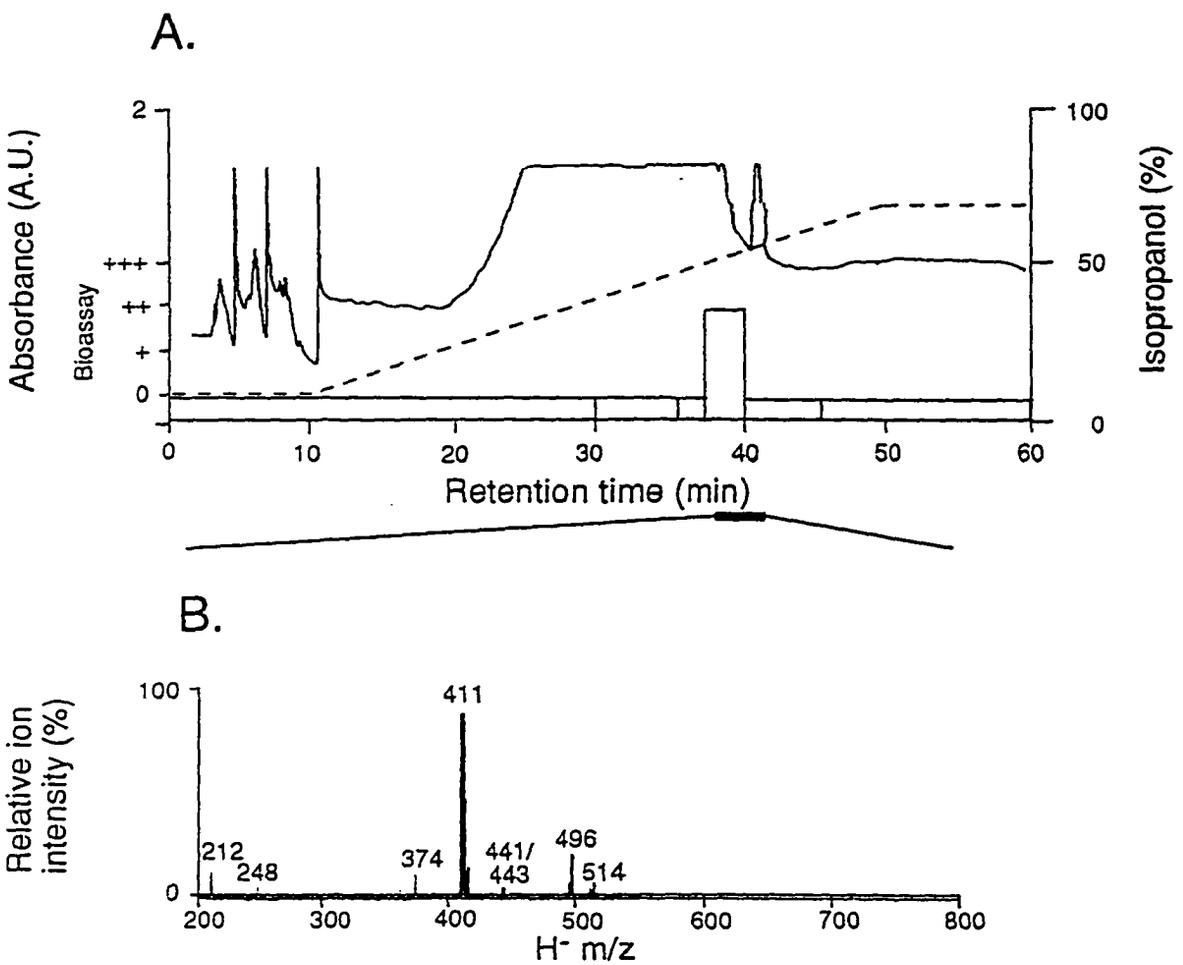

A.
Absorbance (A.U.)
Bioassay
+++
++
+
0
2
100
50
0
Isopropanol (%)
0
10
20
30
40
50
60
Retention time (min)
B.
Relative ion intensity (%)
100
0
411
212
248
374
441/
443
496
514
200
300
400
500
600
700
800
H⁻ m/z

Fig. 26. Mature testes negative ion microbore LC/ESI-MS chromatography of fraction 40 of the HPLC separation of the mature testes extract shown in Fig. 25. A). Total ion current from the LC/ESI-MS. B). Mass spectra of the two major peaks observed in A. C). Extracted mass spectra for negative ions m/z 374 and 411. Separation was on a Vydac 218TP51 column with 25% acetonitrile containing 0.1% acetic acid pumped at a flow rate of 30 μl/min.

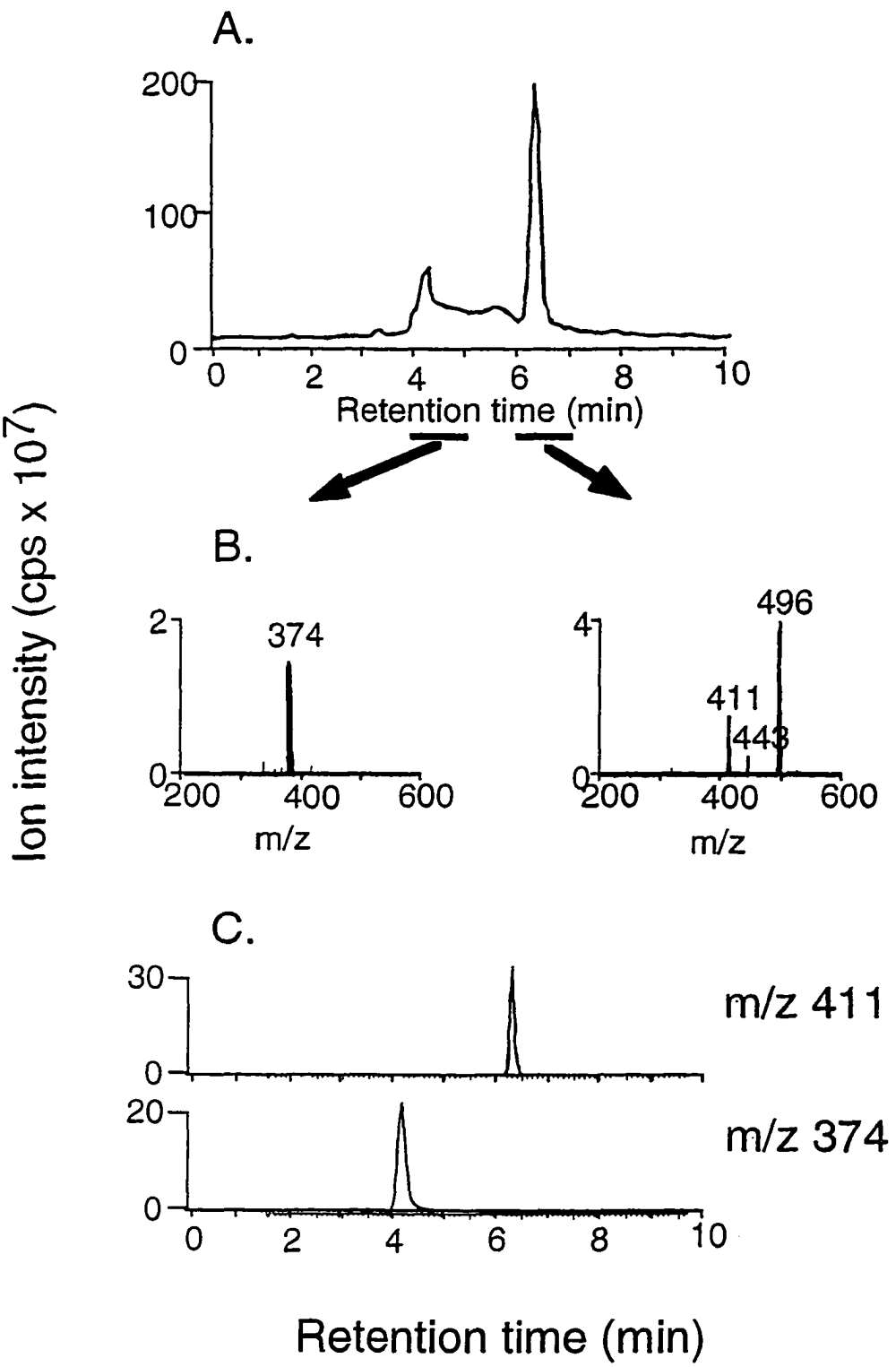

A.
200
100
0
0
2
4
6
8
10
Retention time (min)
Ion intensity (cps x 10^7)
B.
2
374
0
200
400
600
m/z
4
496
411
443
0
200
400
600
m/z
C.
30
0
m/z 411
20
0
m/z 374
0
2
4
6
8
10
Retention time (min)

Fig. 27. Immature testes negative ion microbore LC/ESI-MS chromatography of fraction 40 of an HPLC separation of an extract of immature testes comparable to that of mature testes shown in Fig. 25. A). Total ion current of the LC/ESI-MS run. B). Mass spectra of the two peaks observed in A. C). extracted mass spectra for negative ions m/z 374 and 411. HPLC conditions were the same as described in Fig. 26.

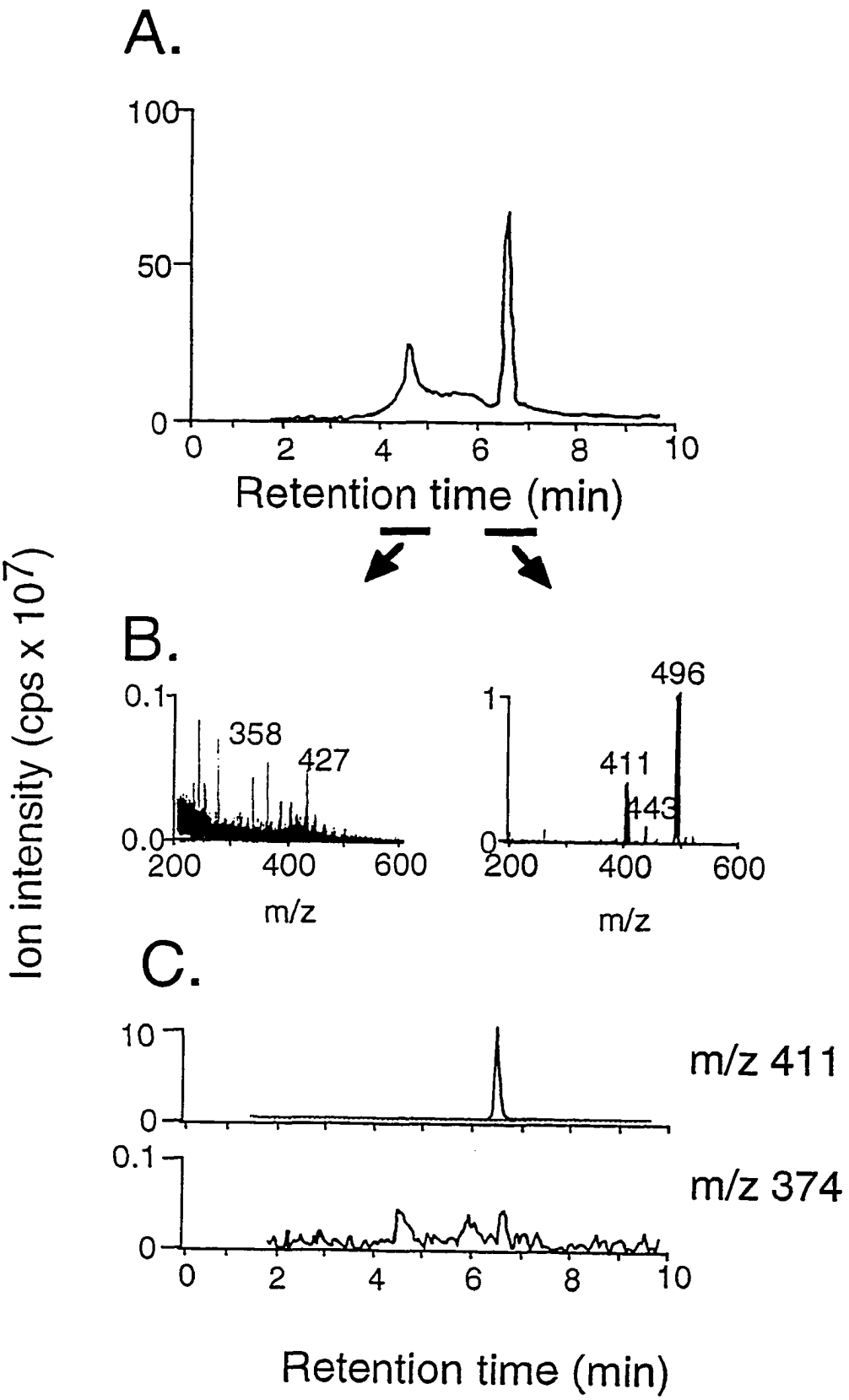
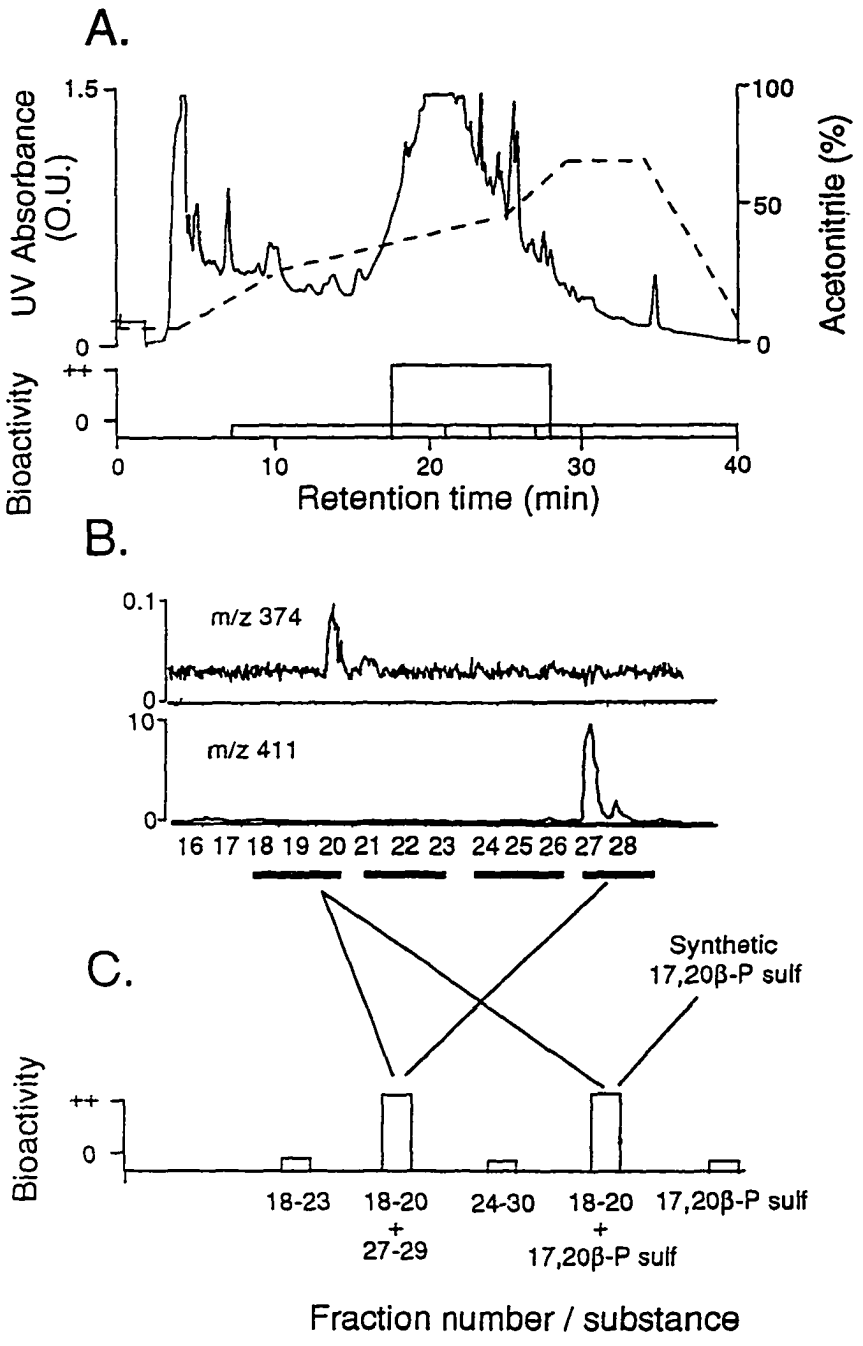

A.
100
50
0
0
2
4
6
8
10
Retention time (min)
B.
0.1
0.0
358
427
200
400
600
m/z
1
0
496
411
443
200
400
600
m/z
Ion intensity (cps x 10^7)
C.
10
0
m/z 411
0.1
0
m/z 374
0
2
4
6
8
10
Retention time (min)

Fig. 28. A). Elution of bioactivity from high pH mini-bore HPLC. Separation was on a Beckman 2.1 x 250 mm ODS column eluted at a flow rate of 0.3 ml/min with a three step program consisting of: 5 min at 10% B; a linear gradient of 10-30% B in 5 minutes; and a linear gradient of 30-60% B in 15 min. Solvent A consisted of 0.125% acetic acid corrected to pH 7.7 with concentrated ammonium hydroxide, whereas solvent B consisted of 70% acetonitrile and 30% solvent A. B). Extracted negative ion mass spectra for ions m/z 374 and 411 of a FIA-MS analysis of sequentially injected fractions of the bioactive region of A. C). Bioactivity of combinations of fractions within the bioactive zone of A and synthetic 17,20ß-P sulphate. Bioactivity is presented as described in Fig. 20.


A.
UV Absorbance (O.U.)
1.5
0
100
50
0
Acetonitrile (%)
Bioactivity
++
0
0
10
20
30
40
Retention time (min)
B.
0.1
m/z 374
0
10
m/z 411
0
16 17 18 19 20 21 22 23 24 25 26 27 28
C.
Synthetic 17,20β-P sulf
Bioactivity
++
0
18-23
18-20 + 27-29
24-30
18-20 + 17,20β-P sulf
17,20β-P sulf
Fraction number / substance

17,20ß-P (10^{-4}M) with the pool of fractions containing the negative ion m/z 374 resulted in a bioactive mixture (Fig. 28 C). Neither the synthetic 17,20β-P sulphate nor the early eluting pool of fractions were pheromonally active on their own in this test.

DISCUSSION

I have purified the herring spawning pheromone with various techniques, resulting in the identification of three principal components in the purified extracts as sulphated steroids: taurocholic acid, cortisol sulphate, and 17α, 20ß-dihydroxy-4-pregnen-3-one (17,20ß-P) sulphate. The 17,20ß-P sulphate was consistently present in the bioactive material, but none of these compounds alone or in combination with one another were bioactive. However, I found that the 17,20ß-P sulphate is bioactive once combined with another, as yet unidentified compound (negative ion of m/z 374), that elutes separately from high pH mini-bore HPLC. My data demonstrate that the herring spawning pheromone consists of at least two compounds, one of which is 17,20ß-P sulphate. This is the first definitive example of a fish pheromone of the "releasing" type that has been shown to consist of individually inactive components. Fish pheromones may consist of mixtures (Sorensen et al., 1988; Resink et al., 1989; Sorensen et al., 1991; van den Hurk and Lambert, 1983), but in most cases studied so far individual components appear to have some activity. In contrast, pheromones of numerous insects are known to consist of several components that may be individually inactive (Roelefs, 1995), and Sorensen (1996) has predicted that the same may be true of vertebrates. Moore and Scott (1992) have also proposed that 17,20ß-P sulphate is part of a multi-component pheromone in the urine of Atlantic salmon, even

though it is inactive as a pheromone on its own (Scott et al., 1994), but proof of this hypothesis is still lacking.

The present work is also the first definitive identification of a component of a releaser-type spawning pheromone of a fish, though prostaglandin F metabolites are thought to comprise the spawning pheromone of goldfish (Sorensen et al., 1995a). The parent steroid of 17,20ß-P sulphate, 17,20ß-P, is likely to be involved in initiation of milt production in the herring (Carolsfeld et al., 1996; Chapter 3), and so 17,20ß-P sulphate could be considered a "hormonal pheromone" comparable to those of other fish (Van Weerd and Richter, 1991). Sulfation of steroids in fish appears to function primarily for metabolic inactivation and elimination (Scott and Vermeirssen, 1994), though in mammals it also may serve to create a reservoir of inactive steroids for reactivation when needed (Hobkirk, 1993). Specific production of sulphated steroids for pheromonal purposes in fish has not yet been demonstrated. 17,20ß-P sulphate is also present in immature testes of herring (Fig. 27), suggesting that the production of this compound specifically for pheromonal signalling is unlikely in this species.

Identification of the components of the herring spawning pheromone was carried out primarily with LC/ESI-MS and MS/MS techniques, and is to my knowledge the first pheromone to be identified in this fashion. My definitive bioassay and the development of protocols that kept bioactive components together until late in the purification scheme were critical in this success, but the electrospray LC-MS and tandem mass spectrometry also proved crucial in permitting the monitoring of underivatized compounds in complex extracts at various steps of purification, particularly in the negative ionization mode. This approach is more objective than the more commonly used procedure of screening known

reproductive hormones of fish for putative pheromonal activity with olfactory recordings, and allows more specific analysis of compounds in crude extracts than does GC/MS. LC/ESI-MS/MS with MRM also permits, in theory, structural elucidation in a fashion analogous to that derived from fragmentation patterns in GC/MS (Johnson and Yost, 1985), but a large databank of mass spectra from electrospray LC/ESI-MS/MS is not yet available. Thus, identification of unknown compounds still requires information from techniques other than LC/ESI-MS/MS, though confirmation of identity is possible once a synthetic candidate is available.

Conjugated and free 17,20ß-P and cortisol immunoreactivity have been reported from herring milt previously (Scott et al., 1991), and sulphated 17,20ß-P is anomolously high in the plasma of males prior to milt production (Carolsfeld et al., 1996; Chapter 3). Sulphated 17,20ß-P was previously eliminated as a pheromonal candidate because it 1) is present in extracts of immature testes, which are inactive; 2) is apparently absent in some bioactive HPLC fractions (results not shown); 3) is insensitive to aryl sulphatase digestion (Scott and Canario, 1992), which was reported earlier to destroy pheromonal activity (Sherwood et al., 1991); and 4) has an HPLC elution time well out of the elution range of bioactivity under some conditions (Carolsfeld et al., 1992). However, since our initial study (Sherwood et al., 1991) I have found that pheromonal activity is not always reduced by aryl sulfatase digestion and in the present work I have confirmed the presence of sulphated 17,20ß-P in purified pheromonal extracts using LC/ESI-MS/MS and MRM.

The sulphate and adjacent hydroxyl group in position 17 of the 17,20ß-P molecule may form a hydrophobic lactone under certain conditions, as indicated by the m/z 97 daughter ion in negative ion MS/MS (Fig. 22), corresponding to the expected mass of a combined sulphate-

hydroxyl moeity. pH-sensitive formation of such lactones by bonding between adjacent hydroxyl groups has been reported for prostaglandin E_2 (Powell, 1987), and has led to a selective isolation protocol based on shifting the compound between organic and aqueous phases of solvent separation systems by altering pH. Likewise, I found that some of the herring pheromone bioactivity is also carried into the organic DCM phase at low pH in protocol D of the present work and can subsequently be recovered by rinsing the DCM with an aqueous borax buffer (pH 9.9) (results not shown). Negative ion FIA/MS suggests that sulphated 17,20ß-P is present in this fraction. Such mechanisms could lead to unpredictable chromatographic performance, as has been reported for sulphated steroids in ion exchange chromatography (Siiter, 1979). This could also explain, to some extent, the broad elution of the pheromonal activity in the various purification protocols I have used (Fig. 18), and insensitivity of 20ß-sulphated pregnens to sulfatase digestion (Scott and Vermeirssen, 1994).

The coincident elution of bioactive components of the spawning pheromone in various chromatographic systems suggests that the unknown component(s) is similar to 17,20ß-P sulphate. A putative second pheromonal component such as this corresponds with the negative ion m/z 374, as yet of unknown identity. Although the negative ionization mode is sensitive for sulphated compounds it also appears to be quite selective, and other pheromonal components may be present that have not yet been detected. The detection limit of the VG Quattro mass spectrometer for taurocholic acid was about 0.05 ng in the negative ionization mode, but only about 0.15 µg in the positive ionization mode. The sensitivity for 17,20β-P sulphate and cortisol sulphate appears to slightly greater in the negative ionization mode, but about the same in

positive ionization (Fig. 22). Considerable UV absorbance is still present in the final purified extract that cannot be ascribed to the relatively few compounds detected by the mass spectrometer. Thus, compounds that do not ionize well with the EIS conditions used may also be present in the pheromonal extracts. Other compounds that have different chromatographic behavior may also take the place of 17,20ß-P sulphate in the pheromonal mixture in some cases, possibly explaining our earlier finding that sulfatase digestion destroys bioactivity (Sherwood et al., 1991).

Other known reproductive pheromones of fish are primarily glucuronated and free steroids, including progesterones and androgens (Scott and Vermeirssen, 1994). Sulphated 17,20ß-P has been proposed as a female priming pheromone in goldfish (Sorensen et al., 1995b) and salmonids (Scott and Vermeirssen, 1994; Scott and Canario, 1992), whereas prostaglandins have been proposed as spawning pheromones in goldfish (Sorensen et al., 1988). However, the present work is the first record of taurocholic acid in milt and testes. As a bile acid, taurocholic acid is likely to be a major component of urine and feces, and may thus be a contaminant from these sources in artificially extruded milt. Bile acids have been proposed as a homing pheromone in salmonids (Hara et al., 1984) and lamprey (Li et al., 1995), and thus also bear further investigation as a potential pheromone of herring.

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CHAPTER 6. CONCLUSIONS

An understanding of the interaction between reproductive physiology, spawning behaviour and pheromonal communication of the Pacific herring will help to refine our understanding of fish physiology, behaviour, and evolution. The herring are considered to be representative of the basal teleost lineage leading to euteleosts (Nelson, 1984) and possess an apparently primitive form of spawning behaviour that is devoid of individual mating but is characterized by a striking pheromonal interaction. This fish could thus be a particularly good model for pursuing comparative work on the evolution of fish reproduction. In this thesis, I, with the help of various collaborators, have made progress in developing the herring model by characterizing the behavioural response of herring to their spawning pheromone, the steroid mechanisms involved in reproductive physiology of the species, the structure of the herring pheromone, and the structure of two types of reproductive proteinaceous hormones of the herring.

I found that the behavioural response to the pheromone is a modal action pattern in that it consists of a series of predictable behavioural events. However, Sorensen (1996) suggests that the response to pheromones can be influenced by experience and physiological state. I searched for factors that influence the intensity of the response, the sensitivity to the pheromone, and the prolongation of the response and found evidence of influence not only by the physiological state, but also by other factors. My working hypothesis was that the degree of sexual maturity and stress would be the prime determinants of how the response is expressed. After first characterizing changes in reproductive steroids associated with maturation in the herring (Chapter 3), I found physiological evidence that the propensity to respond to the pheromone can, in fact, be related to small differences in

maturity, but is not clearly related to stress (Chapter 4). Evidence from other fish species suggests that responses to reproductive pheromones are generally limited to fish of a particular reproductive stage (Sorensen, 1996), but my results are the first to indicate a similar influence within a single maturational stage. Tavolga's (1956) description of pheromone -induced courtship of the frillfin goby is the only other research of which I am aware, that examines individual variability of the pheromone response amongst fish of the same maturational stage. The pheromone response of this species is similar to that of the herring in that some individuals will not respond readily to the pheromone, despite being indistinguishable from other ripe male fish. Tavolga (1956) equates this characteristic to a psychological "set". As with the herring, the "set" towards responding to the pheromone can change within a period of several hours in the frillfin goby as well. Tavolga (1956) found that the "set" of the frillfin goby can be modified by participation in extensive courtship elicited with the assistance of visual cues. In the same study, visual cues were found to be effective in prolonging pheromone-induced courtship of this goby beyond the 30 min observed with pheromone stimulus alone. We have also found that accessory cues other than the spawning pheromone are apparently capable of eliciting spawning behaviour of the herring, and are important in ensuring a prolongation of pheromone-induced spawning (Chapter 2). Some of these accessory cues may be olfactory and pheromonal in nature, as in the shallow-water induced spawning, while others may be more complex, as with the effects of *Macrocystis* kelp on spawning in larger tanks. Future work will focus on defining these accessory cues more clearly and discovering how they interact with the response to the spawning pheromone.

Additional major contributions I have made with this thesis work are the demonstrations that the pheromone consists of at least two components and

the identification of one of these components (Chapter 5), the description of seasonal changes in plasma levels of reproductive steroids of the herring, and the structural identification of principal proteinaceous hormones involved in the regulation of sexual maturation (GnRHs and GtH II-β; Appendices A and B). These will be important tools for future research on the function of the herring spawning pheromone in permitting more precise experimentation on the effects of maturity on the production of, and response to, the pheromone.

We found that one of the components of the spawning pheromone of herring is a sulphated metabolite of 17,20β-P, the steroid we believe to be responsible for initiation of milt production. However, we found that at least a second component is needed to make the pheromone active. A likely candidate for this second component was partially characterized. These results address two of the key questions posed at the start of the thesis. First, the spawning pheromone of herring is at least in part a "hormonal pheromone" comparable to those found in other fish. Second, specificity as to the species and timing of the herring spawning pheromone may be at least partly provided by the multiple components in the active pheromone. Once the second component is identified, the herring pheromone model will be useful in addressing the question of whether pheromonal communication in fish is or is not true communication with specialized signalling and reception mechanisms. For example, further research could address the questions of if olfactory hypersensitivity is present for individual components of the pheromone or only for the mixture, if other compounds are active as pheromonal components, and if pheromone production is regulated specifically for communication.

The physiological aspects of reproductive biology I describe for the herring (Chapter 3) show that the basic patterns of steroid abundance in the

blood during maturation are similar to those described for other fish species. However, future research on distinctive features may provide insights to the evolution of metabolism of reproductive steroids in teleosts and of the relationship between these metabolic pathways and pheromonal communication. Three features of reproductive steroid levels of herring that may provide useful insights are 1) low plasma levels of the free form of the likely maturation-inducing steroid (17,20β-P) in females; 2) high levels of 17-P and the 5β-reduced metabolite of 17-P (3,17-P-5β); and 3) high levels of glucuronated steroids. We have interpreted these results as indicating that 17,20β-P is an evolutionarily primitive maturation inducing steroid (MIS) and that glucuronation is a primitive mechanism for deactivating reproductive steroids in teleosts. However, we suggested that low circulating levels of the MIS appeared several times during teleost evolution, and that the metabolic mechanisms employed in maintaining the MIS low may have led to a variety of MIS-related novel steroids that could be recruited for informative pheromone function. Interestingly, we found no evidence so far of the pheromonal use of glucuronated or 5β-reduced steroids in the herring, though these are the types of steroids considered to be prime pheromonal candidates in some teleosts (Scott and Canario, 1987). Rather, a sulphate-conjugated metabolite is part of the spawning pheromone. It is unclear if this indicates that sulphation functions as a specific pheromone-producing mechanism, and is thus more restricted in activity than glucuronation. Future work should focus on: 1) studies with radio-labelled precursors to confirm whether other steroids that we have not yet identified are important in herring maturation, 2) studies on conjugation rates of the various steroids, and changes in these rates, to see if pheromone-specific production is present, and 3) measurement of olfactory sensitivites of herring to the various

conjugated steroids to indicate how sensory sensitivity and pheromonal function are related in this species.

Knowledge of the structures of the GnRH molecules present in the herring (Appendix A) and the consequent availability of synthetic versions will provide the opportunity to experimentally induce advances in maturity with specific forms of GnRH that are biologically relevant. Specifically, the herring is the most basal teleost yet found to have three GnRH forms, so investigation of the differential function of the three forms is of interest and may have implications for production of, or response to, pheromones. Based on our results with herring, we have suggested that salmonids have recently lost a form of GnRH in their evolution and have found some evidence from primitive salmonids to support this view. Conjugation of reproductive steroids is also less pronounced in salmon than in most other fish studied. It would be of interest to determine if these low levels of conjugation are part of a physiological deficit resulting from the loss of a GnRH form, and, conversely, if the homologous GnRH form in other fish species is directly involved in pheromone production.

The isolation of a cDNA for the GtH II-ß subunit of herring (Appendix B) will lead to the development of an immunoassay for measurement of this "maturational" form of gonadotropin (Swanson, 1992). This GtH assay will be novel amongst fish GtH assays in that it can rely on a gonadotropin standard that is produced *in vitro*, rather than being repeatedly extracted from herring pituitaries. The cost of the pure peptide is thus reduced, both in terms of the number of fish that are sacrificed and in terms of the labour of purification, and, due to the relatively unlimited supply of the pure peptide, non-radioactive assays will become feasible. Future work would apply similar isolation procedures to a cDNA for GtH I-ß. Assays for both GtH proteins and for steroids important in reproduction (characterized in Chapter 3), will

provide tools which should lead to a much broader understanding of herring reproductive physiology. This may be particularly interesting because of the basal position of the herring line in teleost evolution.

Discovery of the GnRH and GtH structures in herring has also provided novel insights on the evolution of these peptides (Appendices A and B). In particular, we presented evidence for the ancient nature of the presence of three GnRH forms; provided alternate hypotheses for the origin of "salmon" GnRH; suggested that recent salmonids have lost a GnRH form; and developed an alternate interpretation of the evolutionary relationships between tetrapod and fish gonadotropins.

In summary, this thesis has made a considerable contribution to understanding the spawning pheromone of the herring. In addition, it has set the stage for development of the herring as a "primitive" teleost model that should be useful for understanding reproductive physiology, fish pheromonal communication and the evolution of both.

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APPENDIX A. PRIMARY STRUCTURE OF A NOVEL FORM OF GONADOTROPIN RELEASING HORMONE OF THE PACIFIC HERRING *CLUPEA HARENGUS PALLASI*[5]

ABSTRACT

Three forms of gonadotropin-releasing hormone (GnRH) were purified from the brain of Pacific herring *Clupea harengus pallasi*, and sequenced using Edman degradation. Two forms correspond with known structures of chicken GnRH-II (cGnRH-II) and salmon GnRH (sGnRH) that are found in many fish. The third form has a primary structure of pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2 and a mass of 1087.9 Da. This novel structure is named herring GnRH (hGnRH). The hGnRH was also purified and sequenced from the herring pituitary, where it was found to be the most abundant immunoreactive form. To date, the herring is the most phylogenetically ancient teleost found to have three detectable forms of GnRH. I suggest that the presence of a third GnRH is an ancestral condition for teleosts, and that some fish species that appear to have only two forms, such as the tetraploid salmonids, cyprinids, and catfish, may have lost one GnRH form. Also, the herring is the most phylogenetically ancient fish found to have sGnRH, demonstrating that the origin of this form of GnRH predates the evolution of the salmonids. Based on comparison of structure and distribution of GnRH peptides, I propose three principal evolutionary lines of

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[5] A version of this chapter has been submitted for publication to J. Mol. Evolu. with co-authors J.J. Powell (current address Aquametrix, Sidney, B.C.), M.Park, W.H. Fischer, A.C. Craig, and J.E. Rivier (Salk Institute, La Jolla, Calif. USA), and N.M. Sherwood (Dept. Biology, UVic.). Dr. Powell purified the peptides, which were then identified by the group at the Salk Institute. J. Carolsfeld participated in the initial conceptualization of the work and, in consultation with Dr. Sherwood, created the current interpretation of the results

GnRH forms: cGnRH-II, sGnRH and mGnRH-like (including hGnRH). Maximum parsimony (PAUP) and neighbor joining (NJ) phylogenetic analyses of neither the 23 known GnRH precursor molecules nor the 12 known mature GnRH peptides clarifies the evolutionary origin of sGnRH. However, the phylogenetic analyses of the mature peptides suggest that the third forms of GnRH, including hGnRH, cluster together with mGnRH, supporting the idea of a common evolutionary origin of these forms.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide best known for its action in releasing gonadotropins from the pituitary of vertebrates (Matsuo et al., 1971, Monahan et al., 1971). The peptide and its precursor are useful models for molecular evolution because the peptide is a reasonable size for protein sequencing and has a central role in reproduction that should dictate strong selective pressure for efficient function, whereas the precursor is large enough for meaningful phylogenetic analysis (Dores et al., 1996). In addition, the molecule has practical significance in controlling reproduction, and so has received considerable attention. Other less well understood functions of GnRH include neuromodulation in various parts of the nervous system (Jan et al., 1983; Leranth et al., 1985) and release of growth hormone from the pituitary in fish (Marchant et al., 1989).

Results of Chapters 2 and 4 suggested that maturity of a herring individual influences the receptivity of that fish to the spawning pheromone. As GnRH plays a central role in the control of sexual maturation, it was felt that identification of the forms present in the brain of this species would be a good first step towards elucidating the control of

this aspect of pheromonal communication. However, the GnRH forms of herring are also of particular interest to the investigation of the evolution of the GnRH molecule, as discussed below, and are the principal focus of this appendix.

Present evidence indicates that GnRH was present early in chordate evolution (Powell et al., 1996b) and is present in multiple forms in most vertebrate species (Sherwood et al., 1994). Eleven of these forms have been sequenced (Powell et al., 1996b), and more may exist. The origin and functional significance of these multiple forms is still controversial. Chicken-II GnRH (cGnRH-II) was first isolated from chicken brains (Miyamoto et al., 1984), but is now known to be the most widely distributed, and possibly the most ancient form in jawed vertebrates (Sherwood et al., 1994). Mammalian GnRH (mGnRH) was first isolated from mammals (Matsuo et al., 1971, Burgus et al., 1972), but is now considered an ancient form because it is present in early bony fish (Lescheid et al., 1995) and amphibians (Conlon et al., 1993), as well as in placental mammals. Salmon GnRH (sGnRH), present in most teleosts studied, and chicken GnRH-I (cGnRH- I), found only in birds and reptiles, are considered to have arisen from mGnRH (Sherwood et al., 1994). Additional forms of GnRH are present in cartilaginous fishes (Lovejoy et al., 1992) and the more advanced perciform fishes (Powell et al., 1994), whereas catfish GnRH (cfGnRH) appears to replace sGnRH in some catfish (Ngamvongchon et al., 1992).

Three principal concerns in the current evolutionary scheme of GnRH relationships are: 1) the origin of sGnRH, 2) the origin of the third GnRH form found in some euteleosts and 3) the functional significance of the multiple GnRH forms. These questions are of interest because: 1) sGnRH is present in most euteleosts investigated, but has not yet been

characterized using chemical sequencing in more primitive fish; 2) current evidence suggests that the presence of 3 forms of GnRH in a single species arose independently in a number of different groups; and 3) although all GnRH forms appear to have a distinctive distribution within the brain, all are effective at eliciting gonadotropin release in fish (Zohar et al., 1995).

The Pacific herring is an extant representative of an early-evolving teleost group (Order: Clupeiformes). Subsequent evolution led to the first euteleosts now represented by the salmonids, carp, and catfish, and the more advanced euteleosts such as the perciform fishes, represented by the seabream *Sparus* spp. (Nelson, 1984). The primary structure of GnRH has been investigated in early and advanced euteleosts, as well as in a pre-teleostean bony fish, the sturgeon. The principal changes in GnRH associated with the evolution of teleosts is the disappearance of mGnRH, the appearance of sGnRH, and, in some groups, the appearance of a third form (Sherwood et al., 1994). At the same time, cGnRH-II is consistently present throughout evolution of cartilagenous and bony fish. As a representative of a basal teleost group, the herring is informative for pursuit of these questions.

MATERIALS AND METHODS

Brains and pituitaries

Brains were collected from 1200 adult Pacific herring *Clupea harengus pallasi* of both sexes about 1 week after spawning. The fish were captured by purse seine in mid-February, 1994, before completing sexual recrudescence, and were held in ocean pens at the Pacific Biological Station (Nanaimo, B.C.) until they spawned in June, 1994.

Brains, with and without pituitaries, were then removed and frozen on dry ice. Isolated pituitaries were removed from a further 50 unspawned mature and 140 spawned herring.

Extraction of GnRH:

Frozen tissues were powdered in a Waring blender with liquid nitrogen and extracted in acidified acetone as described earlier (Ngamvongchon et al., 1992). After filtration, lipids and excess acetone were removed by repeated extractions of the filtrate with petroleum ether; then the remaining acetone was removed from the crude extract with vacuum centrifugation.

Purification of GnRH:

Chromatographic purification of GnRH from the crude extract of herring brains involved six separate steps. The extract of pituitaries was purified in the same manner except that the first and third steps were not used (Table 12). Preparative chromatography was carried out using a Beckman model 166 HPLC pump with a flow rate of 1 ml/min. Fractions of 1 ml were collected from each chromatography step in polyallomer tubes (Evergreen Scientific, Los Angeles). Aliquots of 10 ml were assayed for immunoreactive GnRH (irGnRH) as described in the next section. Residual contaminating irGnRH on the column was tested prior to purification of GnRH by collecting and assaying fractions of a blank run with the first program in Table 12.

For the first step of purification (Table 12, step A), the crude extract of herring brains was diluted 1:1 with HPLC-grade water and loaded onto a column of ten C_{18} Sep-Pak cartridges (Waters, Milford, MA) with a peristaltic pump. The extract was eluted from the column with a linear

gradient of solvent B (80% ACN in 0.05% aqueous trifluoroacetic acid; TFA) from 5 to 65% B in 60 min, after 5 minutes with initial conditions of 5% B.

Fractions from the Sep-Pak column that contained irGnRH were combined, reduced in volume by vacuum centrifugation, and applied to a Supelco C_{18} HPLC column with a 1 ml injection loop in 6 aliquots of 800 ml at 2 minute intervals. Elution, after 10 minutes at initial conditions, was carried out with a linear gradient from 17% ACN in aqueous 0.25M triethylammonium formate (TEAF; pH 6.5) to 24% ACN, followed by isocratic elution with 24% ACN, both with the same aqueous modifier (Table 12; step B). Three distinct groups of fractions with irGnRH were identified by elution position in the purification of brain tissue. These were pooled separately, reduced in volume on a vacuum centrifuge, and subjected individually to further HPLC purification. Only the earliest eluting peak of irGnRH of the pituitary extract was purified beyond this step.

In step C of the purification, based on Rivier (1978), each pool of irGnRH from purification step B was concentrated with vacuum centrifugation and applied to the same Supelco HPLC column as in step B in separate HPLC runs. Elution was carried out with a bipartite linear gradient of ACN with aqueous 0.13M triethylammonium phosphate (TEAP; pH 2.5) after 5 min at initial conditions of 5% ACN. The first of these gradients, from 5% to 20% ACN in 10 min, was followed by a shallower gradient from 20% to 50% ACN in 30 min.

For step D, irGnRH fractions of step C were pooled, concentrated, and applied to the same Supelco HPLC column as before. Elution in this step was carried out with the same mobile phases as step A, but with the bipartite gradient of step C. Step E, using the pooled and concentrated

Table 12. Chromatographic Purification of GnRH from herring brains and pituitaries

Purification Step[1]		Column			Mobile Phase[2]		Sequential elution conditions			
Brains	Pituitaries	Type	Packing	Size (cm)	A	B	Initial %B	Final %B	Time[3] (min)	Flow rate (ml/min)
A	-	Waters, Seppak	C_{18}	1x13	0.5% TFA	0.5%TFA; 80% ACN	5	5	5	1
							5	65	60	
B	B	Supelco	C_{18}	0.45 x 25	0.25M TEAF, pH 6.5	100% ACN	17	17	10	1
							17	24	7	
							24	24	43	
C	C	Supelco	C_{18}	0.45 x 25	0.13M TEAP, pH 2.5	100% ACN	5	5	5	1
							5	20	10	
							20	50	30	
D	-	Supelco	C_{18}	0.45 x 25	0.05% TFA	80% ACN; 0.5% TFA	5	5	5	1
							5	20	10	
							20	50	30	
E	E	Vydac	Phenyl	0.45 x 25	0.05% TFA	80% ACN; 0.5% TFA	5	5	5	1
							5	20	10	
							20	50	30	
F	F	Vydac	C_{18}	0.01 x 25	0.1% TFA	80% ACN; 0.1% TFA	5	60	55	0.05

[1] Purification steps; each line indicates sequential steps of the elution program

[2] Balance of indicated mixture consisted of HPLC water. Abbreviations: ACN, acetonitrile; TFA, trifluoroacetic acid; TEAF, triethylammonium formate; TEAP, triethyl ammonium phosphate.

[3] Time to attain final %B from start of substep of HPLC program

irGnRH fractions from step D, was carried out on a Vydac 219TP54 phenyl column with the same mobile phases and elution program as step D.

irGnRH fractions of each GnRH purification from step E were pooled, concentrated, and shipped frozen to the Salk Institute. Final purification (step F) was then carried out on a microbore C_{18} column eluted with a single linear gradient from 5% B (80% ACN in aqueous 0.1% TFA) to 60% B in 55 minutes.

Radioimmunoassay (RIA)

During the purification of GnRH herring extracts, 10 µl aliquots of each 1 ml fraction were assayed for irGnRH with methods described previously (Sherwood et al., 1991). Antiserum GF-4 (1:25,000 final dilution) was used to detect irGnRH, with mGnRH used as the iodinated tracer and standard. The GF-4 antiserum detects several known forms of GnRH (Sherwood et al., 1986). The limit of detection (B/Bo=80%) for the GF-4 assay was 10 pg. Where tracer binding was outside assay limits (B/B_O=20%), 10 µl aliquots were diluted serially and the value closest to B/Bo=50% was used for analysis.

Sequencing of peptides

Peptides in the three purified irGnRH components from herring brain extraction and one purified irGnRH component from herring pituitaries were sequenced using a Model 470A Applied Biosystems Protein Sequencer. Sequencing was initially attempted on 10% of each sample. Failure of this sequencing indicated a blocked N-terminus. Subsequent sequencing was carried out on the remaining material after digestion with

pyroglutamate aminopeptidase and microbore HPLC purification, as detailed earlier (Fischer and Park, 1992).

Mass Spectrometry

The intact molecule mass of hGnRH was determined on a Bruker Reflex time-of-flight instrument using an accelerating voltage of 31kV and a reflectron voltage of 30 kV (100 MHz digitizer). The sample was applied to a thin layer of α-cyano-4-hydroxy-cinnamic acid, allowed to dry and rinsed with water before analysis. To establish whether the C-terminus of the peptide was amidated, esterification with methanolic HCl monitored with matrix assisted laser desorption ionization-mass specrometry (MALDI/MS) was used (Fischer and Craig, 1994; Powell et al., 1995). Control reactions were carried out with bombesin and bombesin free acid, where a mass shift of 14 Da was observed only for the free acid form.

Phylogenetic analysis

Maximum parsimony and neighbor joining (NJ) phylogenetic trees of GnRH precursors and mature GnRH peptides were constructed with PAUP version 4.0.0d49 (D.L. Swofford, unpublished update of Swofford, 1993). Maximum parsimony phylogenetic trees were constructed of intact peptides using a heuristic search algorithm while Neighbor Joining (NJ) phylograms were constructed based on mean character differences. Bootstrap analyses (100 replicates) were carried out on each tree using the same algorithm.

RESULTS

irGnRH from herring brains

Step A of the purification with the Sep-Pak column resulted in a broad elution of 3.8 mg of irGnRH (Fig. 29 A). All of these fractions were pooled and carried to step B. HPLC purification of this material in step B resulted in three distinct peaks of irGnRH that were designated sequentially according to elution time as herring I-III (Figure 29), and were pooled individually. Each pool was carried through the subsequent purification steps individually (presented as composite graphs in Figs. 29 C-E), resulting in 0.12, 0.45, and 1.04 mg of herring I, II, and III irGnRH, respectively, at the end of step E.

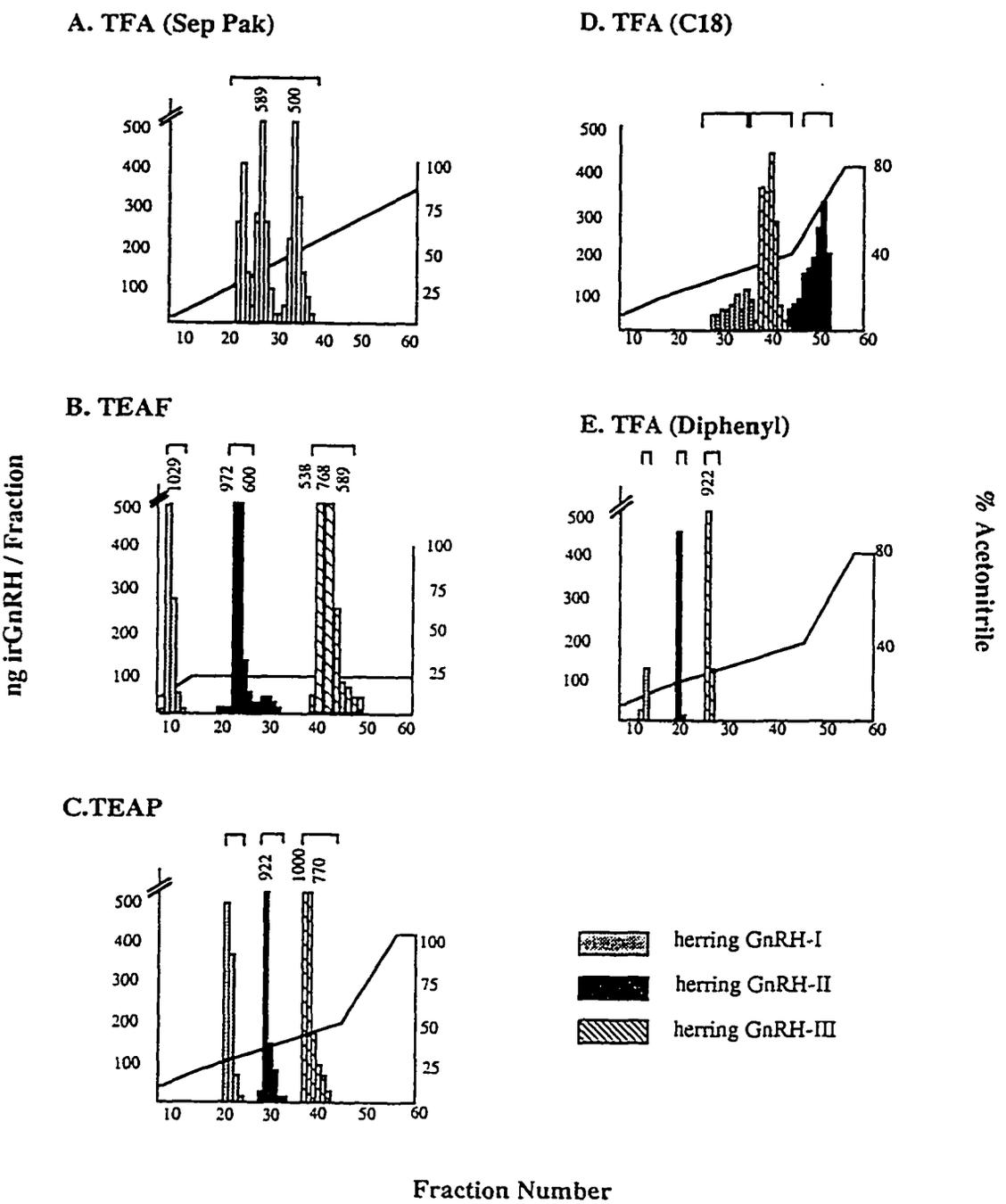
irGnRH from herring pituitaries

The purification of the herring pituitary extract included only steps B, C, E, and F of the purification protocol (Table 12). Three peaks of irGnRH eluted in step B (Fig. 30A), comparable in elution position to those of the brain extract, but only herring I irGnRH was sufficiently abundant (7.5 ng) to carry further in the purification (Fig. 30B). After step E of the purification, 3.0 ng of herring I irGnRH remained (Fig. 30C).

Sequence and mass spectral analysis

The following three amino acid sequences were deduced from the combination of pyroglutamyl aminopeptidase digestion and Edman degradation of the purified immunoreactive components:

Fig. 29. Elution of irGnRH during chromatographic purification of an extract of herring brains. Letters indicate purification steps as outlined in Table 12. GnRH-immunoreactivity of fractions is indicated by bar graphs; different bar graph patterns indicate separate HPLC purification of the three areas indicated in step B. The fractions that were pooled and carried on for further purification are indicated by horizontal brackets over the bar graphs in each step. Lines indicate the acetonitrile concentration of the mobile phase (see Table 12 for details of elution programs).


A. TFA (Sep Pak)
589
500
B. TEAF
1029
972
600
538
768
589
C.TEAP
922
1000
770
D. TFA (C18)
E. TFA (Diphenyl)
922
ng irGnRH / Fraction
% Acetonitrile
Fraction Number
herring GnRH-I
herring GnRH-II
herring GnRH-III

herring I: [pGlu]-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly
herring II: [pGlu]-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly
herring III: [pGlu]-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly.

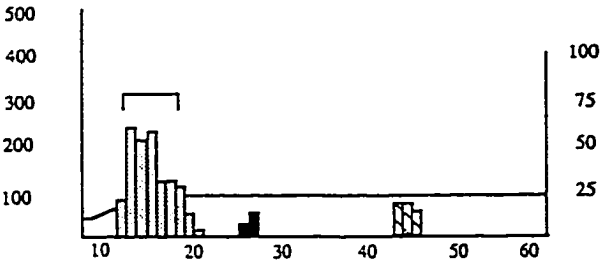
Figure 31 shows the MALDI mass spectrum of the untreated herring GnRH in which an intense species was observed at m/z 1087.9 (compared with thecalculated monoisotopic $[M+H]^+$ of 1087.51 Da for the sequence of hGnRH I shown above). Treatment with methanolic HCl did not result in a mass shift, indicating that the C-terminus was amidated. This structure has not been reported before for a GnRH molecule and therefore I have named it herring GnRH (hGnRH), following the convention of naming a GnRH after the organism from which the primary structure is first determined. The deduced sequences and the elution characteristics during purification of herring II and herring III corresponded to those previously described for chicken GnRH II (Miyamoto et al., 1984) and salmon GnRH (Sherwood et al., 1983). Sequencing of the irGnRH form purified from herring pituitary yielded the same sequence as herring I isolated from the herring brain.

Phylogenetic analysis

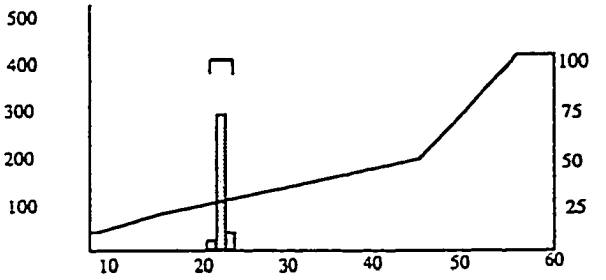
Two equally parsimonious trees were found in the heuristic search of the GnRH precursor sequences. A strict consensus of these two trees (Fig. 32 A) retained substantial structure with strong bootstrap support for most clades. A principal basal grouping consisted of all fish and amphibian GnRH precursors (including that of mGnRH of the frog), separate from the GnRH precursors of mammals and chicken. Within this fish-amphibian clade, the precursor molecules of each form of GnRH

Fig. 30. Elution of irGnRH during chromatographic purification of an extract of pituitaries of herring. Details of purification steps are outlined in Table 12. Only the first peak of irGnRH was carried further in the purification. Patterns and symbols are the same as shown in Fig. 29.

A. TEAF



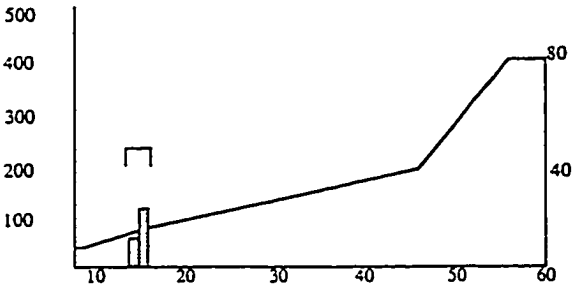
B. TEAP



ng irGnRH / Fraction

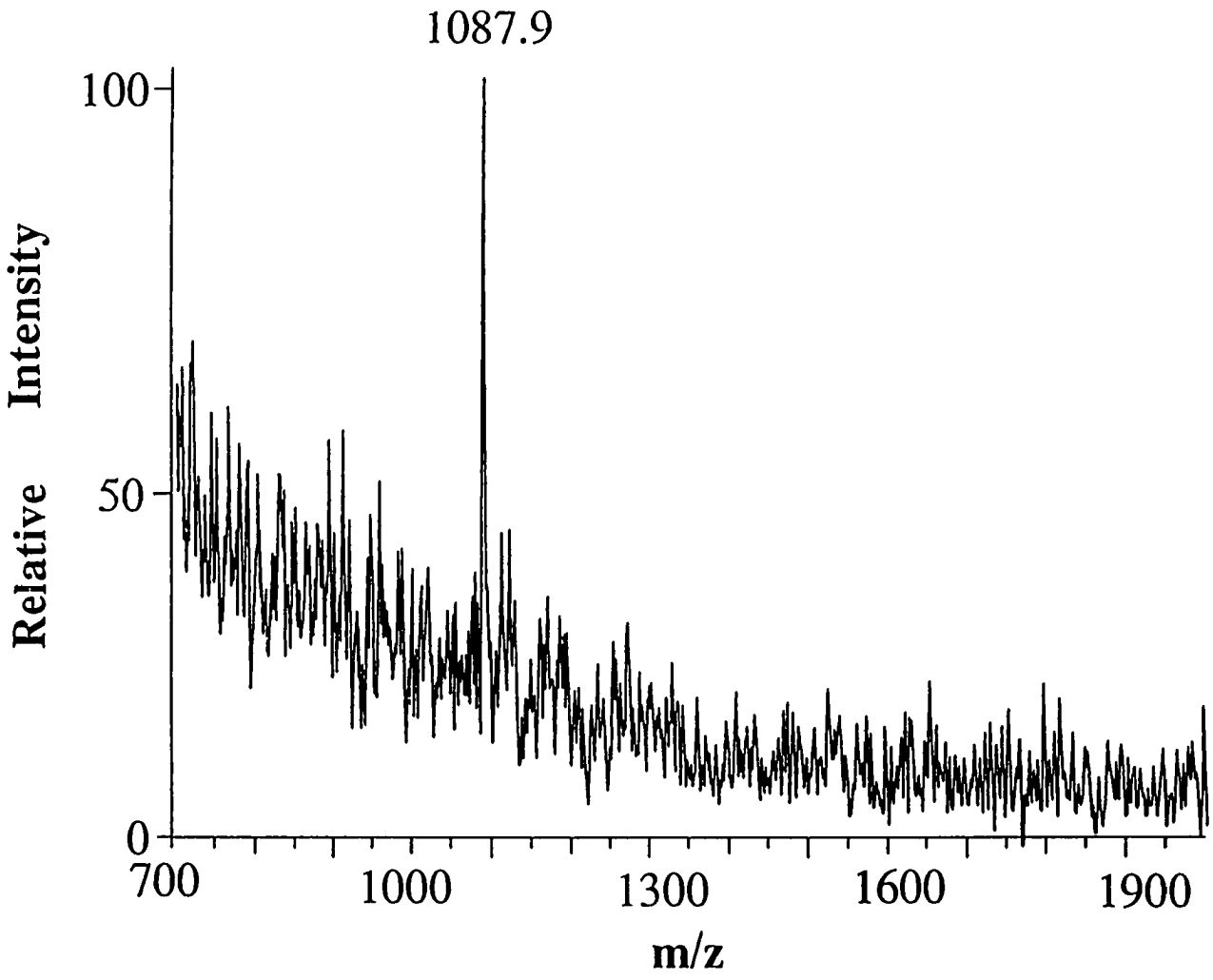
% Acetonitrile

C. TFA (Diphenyl)



Fraction Number

Fig. 31. Matrix assisted laser desorption ionization (MALDI) mass spectrum of hGnRH, showing the signal from the purified peptide (m/z 1087.9). Vertical axis indicates relative intensity of signal, while horizontal axis indicates mass/charge ratio.

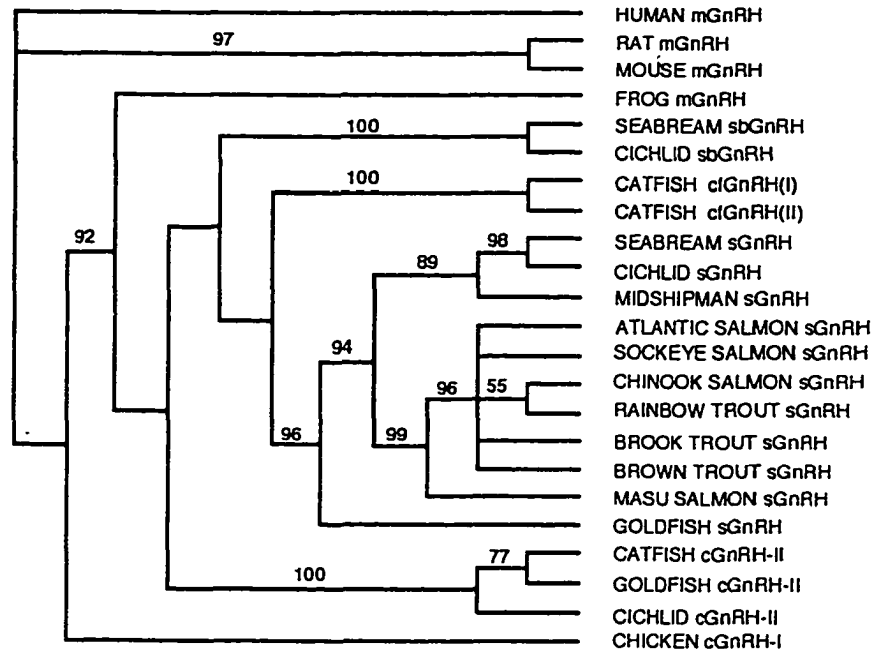

1087.9
100
50
0
Relative Intensity
700
1000
1300
1600
1900
m/z

represented (sbGnRH, cfGnRH, sGnRH, and cGnRH-II) formed the principal groups with good bootstrap support. Additional structure in the tree that had bootstrap support of over 50% was primarily among the sGnRH precursors, reflecting recognized phylogenetic groupings. The NJ analysis provided a very similar tree to that of the maximum parsimony analysis, but with greater bootstrap support for sub-groupings of the GnRH precursors within the fish-amphibian clade. cGnRH-II precursors in the NJ analysis form a group apparently more closely related to the precursors of other fish GnRH forms than in the maximum parsimony analysis, but this distinction has poor bootstrap support (59%). However, the mGnRH precursor of the frog is separated from the fish GnRH precursors to a greater extent in the NJ analysis than in the maximum parsimony analysis.

The heuristic search for maximal parsimonious phylogenetic trees of the mature GnRH peptides (10 amino acids) resulted in 464 equally parsimonious trees of 19 steps (CI=0.947, HI=0.053, RI=0.9, RC=0.853). A strict consensus of these trees collapsed all branches, leaving no structure. However, a 50% majority rule tree retained two clades (Fig. 33), one of hGnRH, cfGnRH, sbGnRH, cGnRH-I and mGnRH and a second of lamprey GnRH-I and tunicate GnRH-II. mGnRH, cGnRH-I and sbGnRH also formed a group within the first of these clades. Only the lamprey I - tunicate II showed boostrap support. A random sampling of 10,000 trees of a heuristic search of the GnRH peptide data matrix resulted in a mean tree length of 25 (S.D.=1.27) and a skewness factor of g_1 = -0.29 ($P<0.001$; Sokal and Rohlf, 1969). The NJ analysis of the same peptides resulted in a greater number of clades, including the first clade of the maximum parsimony tree (mGnRH clade). Only the mGnRH

Fig. 32. Phylogenetic relationships of GnRH preprohormone molecules as deduced from an A) maximum parsimony analysis (PAUP) and B) neighbor joining (NJ) analysis of known sequences. The presented PAUP tree is a strict consensus tree of 2 equally parsimonious trees of a heuristic search (consistency index = 0.86, retention index = 0.87, rescaled consistency index = 0.75) (Swofford, 1993). Bootstrap values of over 50% (100 replicates) have been added for both the PAUP and NJ trees. Branch lengths of the NJ tree are proportional to the estimated evolutionary distance from branch points. Lower case initial letters indicate GnRH form, while upper case names indicate the organism from which the cDNA was isolated. Abbreviations: m, mammalian; sb, sea bream; cII, chicken II; cf, catfish; s, salmon; cI, chicken I. References for sequence data are: m-Human (Seeburg and Adelman, 1984), m-Rat (Adelman et al., 1986), m-Mouse (Mason et al., 1986), m-Frog (Hayes et al., 1994), sb-Sea bream (Gothilf et al., 1995), sb-Cichlid (White et al., 1995), cII-Catfish (Bogerd et al., 1994), cII-Goldfish (Lin et al., 1996), cII-Cichlid (White et al., 1995), cf-Catfish I and II (Bogerd et al., 1994), s-Sea bream (Okuzawa et al., 1994), s-Cichlid (White et al., 1995), s-Midshipman (Grober et al., 1995), s-Sockeye salmon (52), s-Atlantic salmon (Suzuki et al., 1992, Klungland et al., 1992b), s-Chinook salmon (Klungland et al., 1992a), s-Rainbow trout (Suzuki et al., 1992), s-Brook trout, s-Brown trout (Klungland et al., 1992a), s-Masu salmon (Suzuki et al., 1992), s-Goldfish (Lin et al., 1996), cI-Chicken (Dunn et al., 1993).

A. PAUP


HUMAN mGnRH
RAT mGnRH
MOUSE mGnRH
FROG mGnRH
SEABREAM sbGnRH
CICHLID sbGnRH
CATFISH cIGnRH(I)
CATFISH cIGnRH(II)
SEABREAM sGnRH
CICHLID sGnRH
MIDSHIPMAN sGnRH
ATLANTIC SALMON sGnRH
SOCKEYE SALMON sGnRH
CHINOOK SALMON sGnRH
RAINBOW TROUT sGnRH
BROOK TROUT sGnRH
BROWN TROUT sGnRH
MASU SALMON sGnRH
GOLDFISH sGnRH
CATFISH cGnRH-II
GOLDFISH cGnRH-II
CICHLID cGnRH-II
CHICKEN cGnRH-I
97
100
100
92
98
89
94
96
55
96
99
77
100

B. NJ

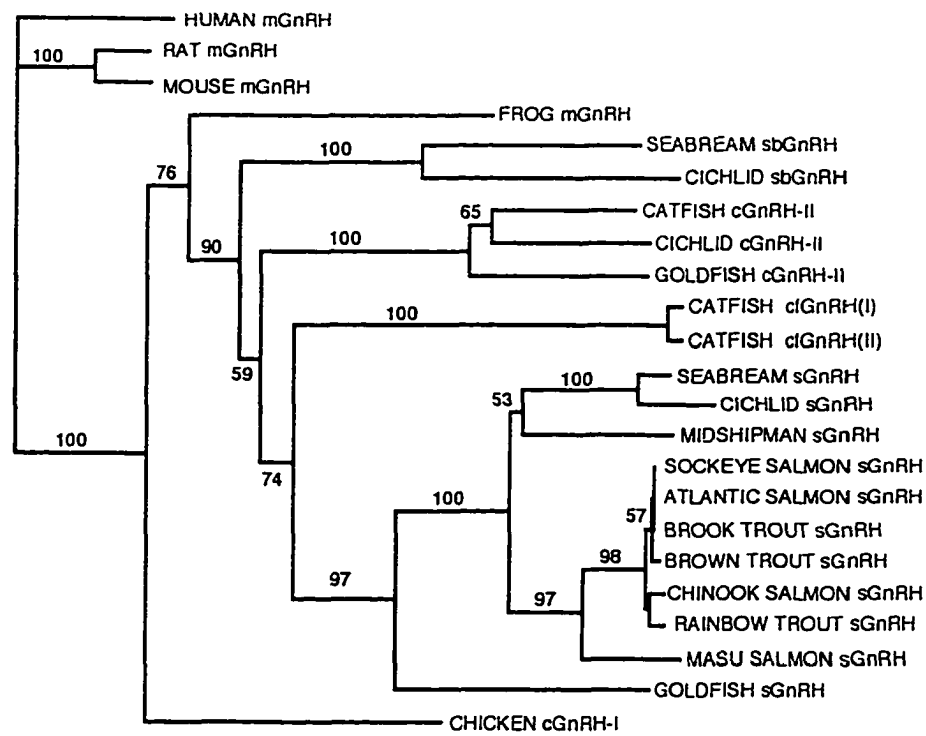
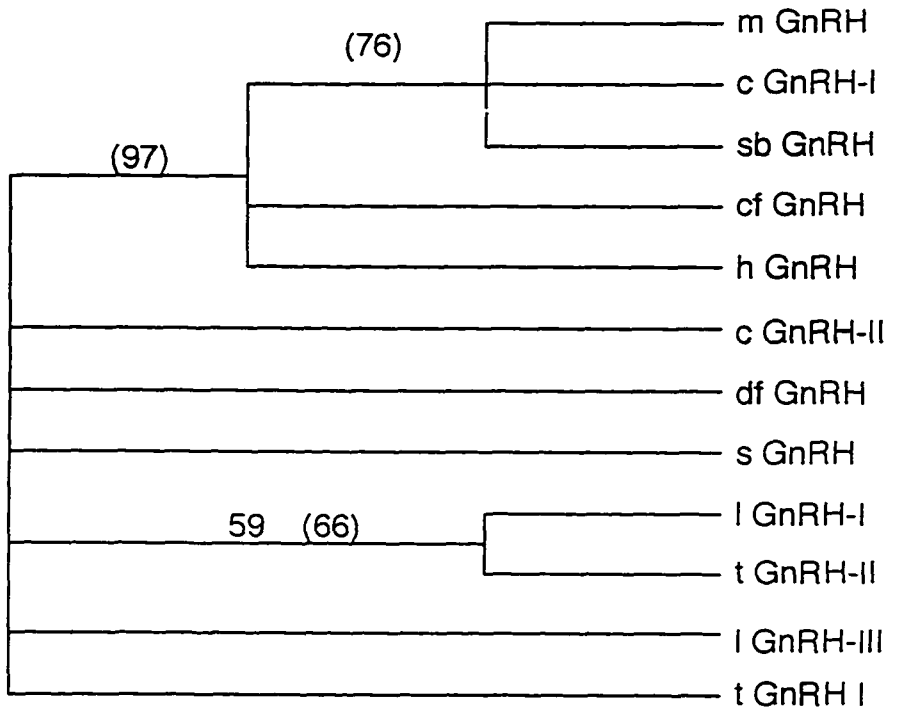
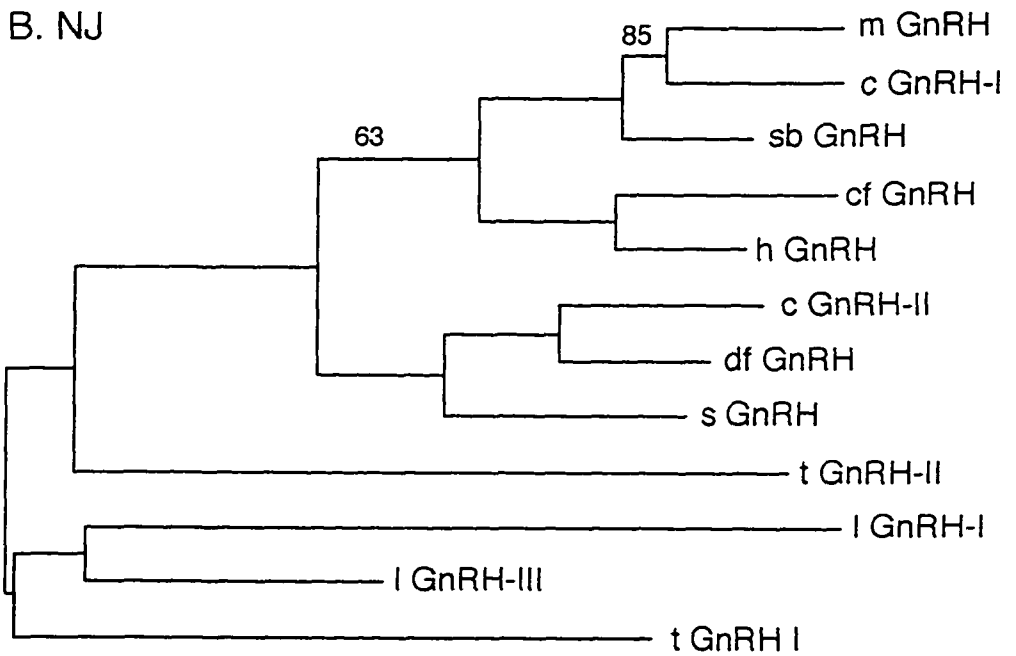

HUMAN mGnRH
RAT mGnRH
MOUSE mGnRH
FROG mGnRH
SEABREAM sbGnRH
CICHLID sbGnRH
CATFISH cGnRH-II
CICHLID cGnRH-II
GOLDFISH cGnRH-II
CATFISH cIGnRH(I)
CATFISH cIGnRH(II)
SEABREAM sGnRH
CICHLID sGnRH
MIDSHIPMAN sGnRH
SOCKEYE SALMON sGnRH
ATLANTIC SALMON sGnRH
BROOK TROUT sGnRH
BROWN TROUT sGnRH
CHINOOK SALMON sGnRH
RAINBOW TROUT sGnRH
MASU SALMON sGnRH
GOLDFISH sGnRH
CHICKEN cGnRH-I
100
100
76
100
65
90
100
100
59
100
53
100
74
100
57
98
97
97

Fig. 33. Phylogenetic relationships of known GnRH forms shown in Fig. 34 deduced with A) PAUP and B) NJ analyses. The presented PAUP tree is a 50% majority rule consensus tree of 464 equally parsimonious arrangements. Numbers in parentheses on the branches of this tree indicate the proportion of trees that contained a particular branch, while other numbers on both the PAUP and the NJ trees indicate the bootstrap support of the particular branch (only values over 50% shown). Lengths of the branches of the NJ tree are proportional to the deduced evolutionary distance from branch points.

A. PAUP (Majority rule)



B. NJ



clade and the subgroup of mGnRH and cGnRH-I showed boostrap support in the NJ analysis.

DISCUSSION

I have identified the primary structure of three forms of GnRH isolated from the brain of the herring: one novel, one identical to cGnRH II, and one identical to sGnRH. In an earlier study, Sherwood (1986) reported the presence of only two forms in the same species, based on chromatographic and immunological evidence. Reexamination of these results indicates that the third form was present in small quantities that were not considered significant at the time.

The newly discovered GnRH form has the lowest mass (1088 Da) and greatest hydrophilicity of any GnRH yet described. It differs from other known forms primarily in positions 5-8 (Fig. 34), an area of the molecule that has previously been identified as the most variable in evolution (Powell et al., 1996b). The histidine and serine in positions 5 and 8, respectively, are probably responsible for making the molecule more hydrophilic than seabream GnRH and catfish GnRH, the two GnRH forms with the most similar structure (90% amino acid identity to hGnRH).

The presence of three forms of GnRH in herring is particularly interesting in that it suggests that three forms of GnRH may have been present early in the teleost stem line when herring evolved. Teleosts are believed to be a monophyletic group (Lauder and Liem, 1983), and since pre-teleostean bony fish have only two forms of GnRH (Lescheid et al., 1995, Sherwood et al., 1991), this has previously been considered the stem condition for teleosts. Three forms of GnRH occur in at least three

orders of fish, but the appearance of the third form of GnRH was thought to be a recent event in evolution of the more advanced euteleostean fish.

The presence of three forms of GnRH in the herring suggests the alternative hypothesis that one form was lost in some later-evolving fish, such as the salmon *Oncorhynchus* spp. (J.F.F. Powell, unpublished; Sherwood et al., 1983), goldfish *Carassius auratus* (Yu et al., 1988) and catfish *Clarias* spp. (Bogerd et al., 1992, Ngamvongchon et al., 1992) (Fig. 35). The euteleost groups represented by these species where a third form is absent, appear to have had a period of tetraploidy in their evolution (Allendorf and Thorgaard, 1984). The loss of a number of chromosomes after the tetraploid event (Allendorf and Thorgaard, 1984) may have resulted in the loss of one of the GnRH genes. A third form of GnRH has been detected by HPLC in a primitive salmonid, the mountain whitefish *Prosopium williamsoni* (J.F.F. Powell, unpublished). Primary structure of this form has not yet been determined, but this result supports the view that more recently-evolved salmonids have lost one GnRH form. Some other diploid fish that have been investigated, such as the molly *Poecilia latipinna* (Coe et al., 1992), medaka *Oryzias latipes* and zebrafish *Brachydanio rerio* (Powell et al., 1996a) so far appear to have only two forms. However, a third form may also be present in these species, but has not been detectable with the limited number of fish used or due to lack of cross-reactivity with available antisera.

The "third" form of GnRH (e.g. sbGnRH) has been hypothesized to be the principal form to induce gonadotropin release from the pituitary in a cichlid *Haplochromis burtoni* (White et al., 1995). This hypothesis is based on the abundance of this form in the pituitary (Powell et al., 1995) and the localization of the sbGnRH-expressing cells in the preoptic area of the brain, with axons terminating in the pituitary. Cells expressing the

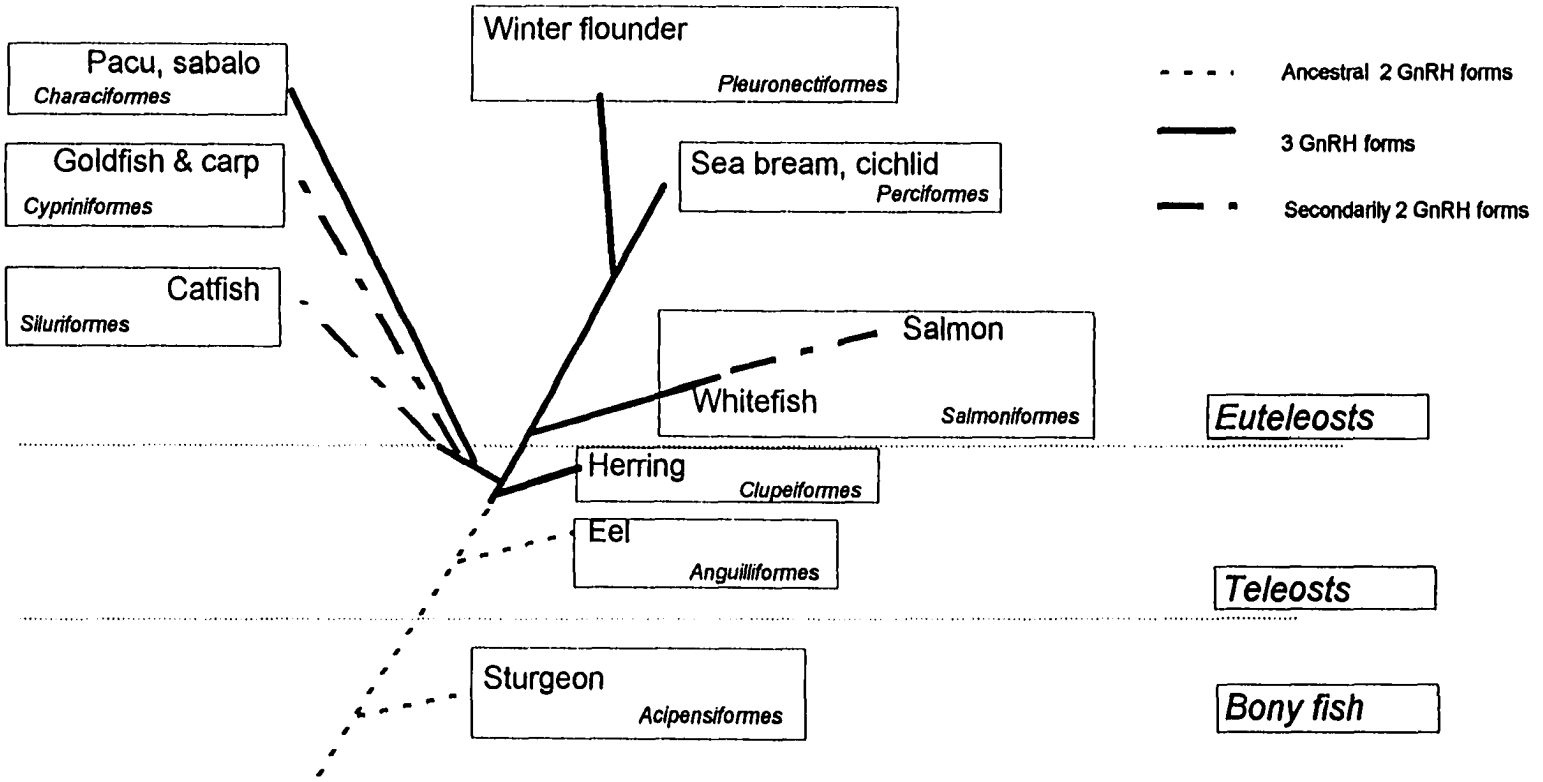
Fig. 34. Comparison of known GnRH structures with herring GnRH. Only amino acid residues different from those in the comparable position of the hGnRH structure are shown.

	Amino acid residue											
	1	2	3	4	5	6	7	8	9	10		
GnRH form												Ref.
Herring	**pGlu**	**His**	**Trp**	**Ser**	**His**	**Gly**	**Leu**	**Ser**	**Pro**	**Gly**	**NH2**	
Catfish	-	-	-	-	-	-	-	Asn	-	-	-	Ngamvonchong et al., 1992; Bogerd et al., 1992
Chicken I	-	-	-	-	Tyr	-	-	Gln	-	-	-	Miyamoto et al., 1983
Chicken II	-	-	-	-	-	-	Trp	Tyr	-	-	-	Miyamoto et al., 1984
Dogfish	-	-	-	-	-	-	Trp	Leu	-	-	-	Lovejoy et al., 1992
Lamprey I	-	-	Tyr	-	Leu	Glu	Trp	Lys	-	-	-	Sherwood et al., 1986
Lamprey III	-	-	-	-	-	Asp	Trp	Lys	-	-	-	Sower et al., 1993
Mammal	-	-	-	-	Tyr	-	-	Arg	-	-	-	Matsuo et al., 1971; Burgus et al., 1972
Salmon	-	-	-	-	Tyr	-	Trp	Leu	-	-	-	Sherwood et al., 1983
Sea Bream	-	-	-	-	Tyr	-	-	-	-	-	-	Powell et al., 1995
Tunicate I	-	-	-	-	Asp	Tyr	Phe	Lys	-	-	-	Powell et al., 1996b
Tunicate II	-	-	-	-	Leu	Cys	His	Ala	-	-	-	Powell et al., 1996b

other two GnRH forms in this species are restricted to the terminal nerve (sGnRH) and the mesencephalon (cGnRH-II). The most abundant irGnRH form in the pituitary of the herring is also the third GnRH form, in this case hGnRH. However, in salmonids where a third GnRH appears to be missing, one of the two remaining forms (sGnRH) is expressed in cells that are found in both the terminal nerve and preoptic area (Suzuki et al., 1992, Bailhache et al., 1994). In catfish, where sGnRH rather than the "third" form appears to be missing, cfGnRH-expressing cells are found in both areas (Zandbergen et al., 1995). Presumably either form can take over the function of the other in their respective areas of the forebrain, so differences in reproductive physiology associated with the loss of one form of GnRH may be subtle.

Structural similarities of the various GnRH forms can also provide some information on likely evolutionary relationships. Lovejoy et al. (1992) and Sherwood et al. (1994) suggest that mGnRH gave rise separately to sGnRH and cfGnRH, in part because mGnRH disappears coincidentally with the appearance of sGnRH in the salmonids and cfGnRH in the catfish. I have shown that both sGnRH and a third GnRH are present in the more basal herring in the absence of mGnRH, suggesting that the gene for mGnRH may have duplicated and given rise to two separate evolutionary lines of GnRH forms, one of which led to sGnRH and the other to hGnRH, cfGnRH, and sbGnRH (Fig. 36). Alternatively, the third form of GnRH resulted from a substitution in the mGnRH gene, whereas sGnRH evolved from a duplication of the cGnRH-II gene. These groupings are also distinguished by the amino acids in positions 5 and 7. Tyrosine in position 5 is characteristic of the proposed mGnRH line of molecules (hGnRH, cfGnRH, sbGnRH, cGnRH-I), whereas

Fig. 35. Proposed evolutionary scheme for GnRH multiplicity in teleosts, showing representative fish species enclosed in boxes indicating taxonomic Orders. See text for references other than for the eel *Anguilla anguilla* (King et al., 1990), pacu *Piaractus mesopotamicus* (Powell et al., 1996c), sabalo *Prochilodus lineatus* (Somoza et al., 1994) and winter flounder *Pseudopleuronectes americanus* (Idler et al., 1987).


Pacu, sabalo
Characiformes
Goldfish & carp
Cypriniformes
Catfish
Siluriformes
Winter flounder
Pleuronectiformes
Sea bream, cichlid
Perciformes
Salmon
Whitefish
Salmoniformes
Herring
Clupeiformes
Eel
Anguilliformes
Sturgeon
Acipensiformes
Ancestral 2 GnRH forms
3 GnRH forms
Secondarily 2 GnRH forms
Euteleosts
Teleosts
Bony fish

tryptophan in position 7 is characteristic of the cGnRH-II line of molecules (cGnRH-II, dogfish GnRH, lamprey GnRH I and II; Fig. 34). sGnRH possesses both tyrosine in position 5 and tryptophan in position 7, and hence is intermediate in character. Tryptophan substituted into position 7 of mGnRH results in increased potency for releasing gonadotropin from the goldfish pituitary (Millar et al., 1989, Habibi et al., 1992), so this may be a substitution that occurred independently of cGnRH-II in the evolution of sGnRH. In addition, the evolution of sGnRH from cGnRH-II would require a change in the regulatory 5' flanking region of the cGnRH-II gene because sGnRH is expressed within the forebrain (olfactory/preoptic areas) whereas cGnRH-II is restricted to the midbrain (White et al., 1995).

Thus the evolutionary origin of hGnRH and sGnRH appears to be in early teleosts, but our structural comparisons do not identify the parent molecule(s). Computer programs have been developed to assist in more sophisticated deductions of phylogenetic relationships between forms of a polypeptide (Swofford, 1993). However, Dores et al. (1996) suggest that phylogenetic trees produced by these programs may be meaningless in the absence of an evolutionary hypothesis, particularly with small peptides. The authors use a comparison of GnRH peptides to illustrate their point. In the present paper, I have developed suitable evolutionary hypotheses for the origins of GnRH forms to explain the patterns presented by Dores et al. (1996). I thus feel it is informative to re-examine such analyses with both the larger precursor molecules (up to 109 aa) and the mature GnRH peptides (10 aa), including additional sequences that became available after the analyses by Dores et al. (1996).

Grober et al. (1995) found that a PAUP analysis of nucleotide bases of 18 GnRH precursor molecules with maximum parsimony resulted in

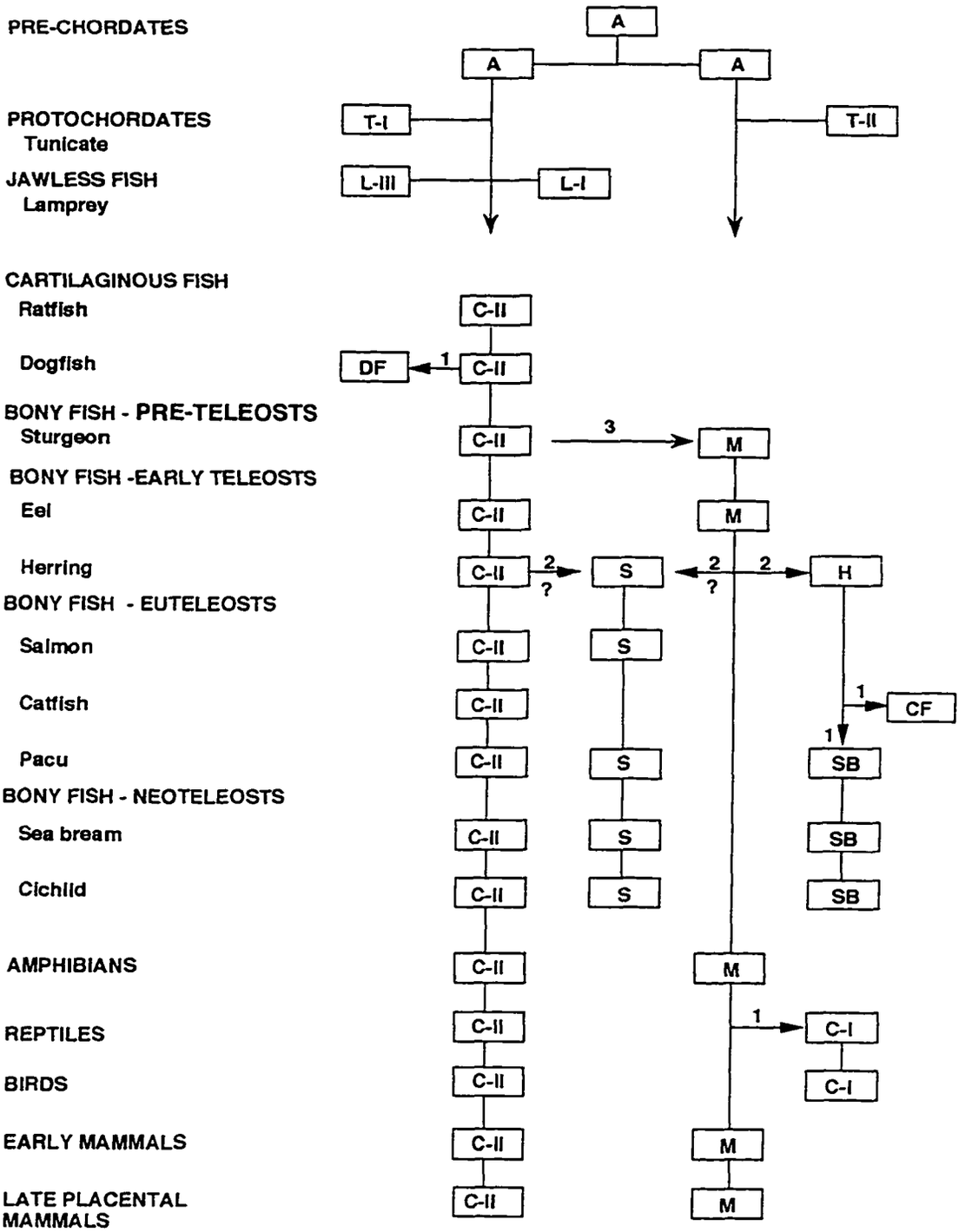
groupings of molecules according to the GnRH form and accepted taxonomic relationships of the donor species; I also have found a similar result with PAUP and NJ analyses of the amino acid sequences of 23 of these molecules. However, the evolutionary relationships between mGnRH, sGnRH and cGnRH-II in our study are not well resolved in any of these analyses, except that 1) sGnRH precursor molecules appear more closely related to those of cfGnRH and sbGnRH than to those of cGnRH-II, and 2) in our analyses a shared evolutionary origin of fish GnRHs and mGnRH may be indicated by the greater affinity of the mGnRH precursor of the frog to the GnRH forms of fish than to those of mammals. Further resolution of evolutionary relationships amongst GnRH forms with this analysis will require knowledge of more precursor molecules because precursors of representatives of only six forms of GnRH have been sequenced.

Twelve forms of GnRH are now known, in contrast to the situation with the precursor molecules. However, the 10 aa sequence of the GnRH peptide may be too short for extensive phylogenetic analysis (Dores et al., 1996, Grober et al., 1995, Sower et al., 1993), so I have taken several steps to improve the information that may be derived from these molecules: 1) I carry out both a parsimony (PAUP) and a distance (NJ) analysis, and 2) I address the specific phylogenetic hypotheses that sGnRH is derived from cGnRH-II and that the "third" GnRH forms are of common evolutionary origin. I also measured the skewness of the distribution of lengths of random trees constructed from the mature GnRH peptide sequences, which is a manner of determining if useful phylogenetic signal is present in the data (Hillis and Heulsenbeck, 1992). Skewness of this distribution was significant, as determined by classical statistics (Sokal and Rohlf, 1981). However, I found that a strict

consensus tree of the maximum parsimony analysis (PAUP; Swofford, 1993) used by Dores et al. (1996) does not effectively resolve the GnRH peptides. The majority rule consensus tree of the maximum parsimony analysis (Fig. 33 A) and the NJ cladistic analysis (Fig. 33 B) resolved a group of five GnRH forms (mGnRH, cGnRH-I, sbGnRH, cfGnRH, and hGnRH), corresponding with the mGnRH line of molecules I present in Fig. 36. Bootstrap support is low, but low bootstrap values can be expected with a large proportion of invariant amino acid positions. sGnRH fails to cluster in the mGnRH group, and appears to be more closely allied to cGnRH-II in the NJ analysis, but these relationships are not sufficiently clear to indicate an ancestral origin of sGnRH in the cGnRH-II line.

With regard to pheromonal communication in the herring, the discovery of three forms of GnRH has several implications. Differential function of the three forms can be hypothesised, as indicated above, with perhaps only one form directly involved in release of gonadotropins. However, sGnRH is likely to be present in the terminal nerve, as it is in the salmon (Suzuki et al., 1992; Bailhache et al., 1994) and the cichlid (White et al., 1995). The terminal nerve was at one time thought to be a specific pheromone detection pathway because of its GnRH content (Demski and Northcutt, 1983), but intracellular electrophysiological recordings have shown this to be unlikely (Fujita et al., 1991). However, the possibility that GnRH influences olfactory sensitivity to pheromones directly through nerve endings in the olfactory epithelium has not yet been clearly tested. I set up an experiment in which I injected synthetic forms of the three native herring GnRH molecules, as well as superactive GnRH analogs, to determine if the various forms had specific actions on gonadal maturation, steroid production and pheromone responsiveness. However,

Fig. 36. Proposed evolutionary relationships of known GnRH molecules, based on structural and distributional comparisons (modified from Sherwood et al., 1994). Numbers on arrows indicate the number of changes in amino acids required for the transition of one GnRH form to another. Abbreviations for GnRH forms are: C-I, chicken I; C-II, chicken II; CF, catfish; DF, dogfish; ; H, herring; L-I and L-III, lamprey I and III; M, mammalian; S, salmon; SB, sea bream; T-I and T-II, tunicate I and II.


PRE-CHORDATES
PROTOCHORDATES
Tunicate
JAWLESS FISH
Lamprey
CARTILAGINOUS FISH
Ratfish
Dogfish
BONY FISH - PRE-TELEOSTS
Sturgeon
BONY FISH -EARLY TELEOSTS
Eel
Herring
BONY FISH - EUTELEOSTS
Salmon
Catfish
Pacu
BONY FISH - NEOTELEOSTS
Sea bream
Cichlid
AMPHIBIANS
REPTILES
BIRDS
EARLY MAMMALS
LATE PLACENTAL MAMMALS
A
A
A
T-I
T-II
L-III
L-I
C-II
DF
1
3
M
2
?
S
H
CF
SB

the fish proved to be too fragile to withstand this type of experimentation, so I do not yet have this information.

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APPENDIX B. ISOLATION AND CHARACTERIZATION OF A CDNA FOR GONADOTROPIN II-β OF PACIFIC HERRING (*CLUPEA HARENGUS PALLASI* L.)[6]

ABSTRACT

The purpose of the study was to identify the pituitary hormone that controls reproduction in Pacific herring (*Clupea harengus pallasi*), a basal teleost lineage. A complete complementary DNA (cDNA) encoding herring gonadotropin (GtH) II-β protein is presented. The cDNA encodes a signal peptide of 24 amino acids and a GtH polypeptide of 149 amino acids. The identity of the herring molecule as GtH II-β is based on 12 conserved cysteine residues, an N-glycosylation site, and a teleost-specific CSGH sequence, thought to be similar to the CAGY motif of mammalian LH-β. Unlike other early evolving-teleosts (Anguilliformes, Salmoniformes, Cypriniformes, Siluriformes), the amino terminus of the mature herring GtH II-β hormone begins with phenylalanine, more typical of lineages that appeared much later (Cyprinodontiformes, Perciformes). Phylogenetic analysis of LH, FSH, and fish GtH II- and I-β subunits shows that groups of GtH II-β subunits of closely related fish species form distinct sister clades to each other and to a clade containing

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[6] A version of this chapter has been accepted for publication in the J. Fish. Biol. with co-authors M.E. Power (current address: Swiss Federal Institute for Environmental Science and Technology, Duebendorf, Switzerland), G.P. Wallis (Dept. of Zoology and Centre for Gene Research, University of Otago, Dunedin, New Zealand), and N.M. Sherwood (Dept. Biology, UVic). Dr. Power carried out the laboratory work of isolating and characterizing the cDNA, whereas J. Carolsfeld was involved in the conceptualisation of the project and carried out most of the analysis of the results, with the assistance of Drs. Sherwood, Koop and Wallis.

LH, FSH, and fish GtH I-β. This result suggests that the β subunit of both LH and FSH may have evolved from the lineage of fish GtH I-β rather than that of GtH II-β.

INTRODUCTION

Gonadotropins (GtHs) are pituitary glycoproteins that play a central role in vertebrate reproduction. In this capacity, they may be important in the regulation of both production of and responsiveness to the spawning pheromone of herring. However, a method of measuring GtH levels of herring does not presently exist. GtH assays are normally carried out with radioimmunoassays, but no cross-reactivity was found with the antibodies we had available (raised against salmon GtH). We thus proceeded to purify herring GtH with chromatographic techniques and screened purified fractions for GtH by sequencing terminal portions of the principal proteins found. This proved to be unsuccessful, so we embarked on an alternative approach of isolating a cDNA of the GtH molecules. This molecular approach has several advantages over traditional chromatographic purification of the mature proteins: 1) less biological starting material is needed, and 2) recombinant GtH can be made from the cDNA for use in immunoassays, rather than relying on further purification from pituitaries. However, full development of an assay for herring GtH and its application was not possible within the time-frame of this thesis, so the initial focus of this work, presented in this chapter, was to investigate the herring GtH molecule from an evolutionary point of view.

Two forms of GtH have been found in the majority of vertebrates investigated: luteinizing hormone (LH) and follicle stimulating hormone

(FSH) in tetrapods and GtH I and GtH II in fish (see Koide et al., 1993). All of these hormones are non-covalently bound dimers of α and β subunits, as are other members of the vertebrate glycoprotein hormone families including TSH and inhibin. In salmon, gonadotropin I is associated with early stages of spermatogenesis and vitellogenesis, whereas GtH II is associated with spermiation and ovulation (Dickhoff and Swanson, 1990). The β subunit of GtH II, rather than the α subunit, is of considerable interest in research on the evolution of vertebrate glycoprotein hormones (Quérat, 1994) because it has greater structural diversity among species. The phylogenetic clustering of the fish GtH II-β subunits reported to date is roughly consistent with recognized phylogenetic relationships in some (Quérat, 1994), but not all (Tsai and Yang, 1995) analyses. Tsai and Yang (1995), using a GENE CLUSTAL program, find that GtH II-β subunits of porgy, tuna, and killifish form a separate cluster from those of other fish, whereas Quérat (1994), using a maximum parsimony procedure, reports a dendrogram of molecular structures more similar to phylogenetic schemes based on morphological characteristics.

Herring, *Clupea harengus* L., is a phylogenetically ancient teleost thought to have branched off between eel and more advanced euteleosts. Thus, analysis of the herring GtH II β subunit could clarify evolutionary schemes of this molecule. Here we describe the isolation and characterization of a cDNA for the β subunit of GtH II of the Pacific herring *C. harengus pallasi* and discuss the relationship between gonadotropin structure and phylogeny.

MATERIALS AND METHODS

Herring pituitary cDNA library construction

A pituitary cDNA library was constructed from Pacific herring pituitaries that were removed just prior to ovulation or spermiation as judged by gonad size and appearance (Hay, 1985). Fifty pituitaries were homogenized in 15 ml guanidinium thiocyanate buffer. Total RNA was extracted by the method of Chomczynski and Sacchi (1987) and polyA+ RNA was isolated by means of oligo deoxyribothymidine (dT) cellulose using the FastTrack mRNA kit (InVitrogen, CA). PolyA+ mRNA (2.4 mg) was used in the construction of a cDNA library using the ZAP-cDNA Synthesis Kit (Stratagene, LaJolla, CA), in which a bluescript vector is used.

Amplification of GtH II-β by PCR

A probe was constructed by polymerase chain reaction (PCR) amplification of a GtH II-β fragment from single-stranded cDNA prepared from herring pituitary messenger RNA (mRNA) (Gibco BRL). Oligonucleotide primers were designed based upon sequences in the chum salmon *Oncorhynchus keta* (Walbaum) molecule corresponding with conserved aa regions in other teleost GTH II-β molecules: GtH forward (5'-CTGTGTCTCTGGAGAAGGAAGGCTG-3') and GtH reverse (5'-GCTCAGAGCCACAGGGTAGGTG-3'). The regions to which the primers hybridized in the herring cDNA are underlined with a dashed line in Fig. 37. Single stranded cDNA was synthesized from 1 mg of herring pituitary mRNA by random priming with an oligo (dT18-20) primer (Pharmacia) and Superscript RT H- reverse transcriptase (Gibco BRL). PCR amplifications were done with the resulting cDNA, 1 x Taq buffer,

200 mM dNTPs, 1.5 mM $MgCl_2$, 5 U Taq polymerase (Gibco BRL), and 20 pmol of each primer in 1 ml of solution. Following incubation of the reactions for 2 min at 94 °C, 35 cycles were carried out for 1 min at 94 °C, 1.5 min at 56 °C and 1.5 min at 72 °C.

The PCR products were run on 1% agarose gels and the band excised and the DNA electroeluted. A portion of the ethanol-precipitated DNA was ligated into pGEM vector-T and electroporated into E. coli XL1-Blue cells (Gene Pulser, Biorad). Plasmid DNA was isolated using Wizard miniprep kit (Promega). The PCR fragment inserts were partially sequenced using M13 Universal Primer and compared to known fish GtH II β subunit sequences to ensure that a GtH fragment had been amplified.

Screening of the herring pituitary library

The PCR probe was labelled with [α-32P] dCTP (1.17×10^8 cpm/ml). A total of 30,000 plaques of the herring pituitary library (1.26×10^8 pfu/ml) were screened. Hybridization was carried out overnight at 45 °C. The nylon membranes were washed at low stringency (successively at 45 °C for 20 min each in 2 x SSC/0.1% SDS, 1 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS), then exposed to Kodak XAR-5 film for 4 days at -80 °C. Positive clones were isolated, repeated and rescreened twice to eliminate contaminating clones. pBluescript phagmid was rescued from the cored third round positive clones with ExAssist helper phage (Stratagene) and cultured in nonsuppressing *E. coli* SOLR.

Sequence analysis

Plasmid DNA from overnight cultures of individual positive clones was isolated using Wizard Minipreps (Promega). Herring DNA was alkaline denatured and sequenced with [α-35S] dATP (Amersham) using

Fig. 37. The cDNA nucleotide sequence of herring gonadotropin II-β subunit (GtH II-β). The herring GtH II-β open reading frame of 149 aa extends from the start codon ATG at nucleotide 1 to the end of the stop codon TAG at nucleotide 450. The stop codon is shown by an asterisk (*). The putative polyadenylation sequence AATAAA is doubly underlined. A polyadenylation tail was observed (not shown). The cDNA open reading frame is in upper-case letters. The 5'UTR (untranslated region) and 3'UTR are in lower-case letters. The putative signal peptide is indicated by a single underline and the region to which the PCR primers hybridized is shown by a dashed underline.

```
-46                                    ctggccacctgaccaactggactgaatgtatctatactctgaaagg

 1  ATG GCC CGT ATC CCA GAG TGC ACT ATT CTG CTT CTT CTG TGT ATG TGT GTA CTG GCT GTA
 1  M   A   R   I   P   E   C   T   I   L   L   L   L   C   M   C   V   L   A   V     20

61  CCT GCA CAG TGT TTC AAC CTG CAG CCC TGT GTA CTA GTC AAC GAG ACC GTG TCC GTG GAG
21  P   A   Q   C   F   N   L   Q   P   C   V   L   V   N   E   T   V   S   V   E

121  AAA GAA GGC TGC CCG AGG TGC CTG GTG TTC CGG ACC ACC ATC TGC AGT GGA CAC TGC CCG
 41  K   E   G   C   P   R   C   L   V   F   R   T   T   I   C   S   G   H   C   P

181  ACC AAA GAG CCT GTG TAC AAA AGC CCA TTC TCC GTG GTG AAC CAG CAC GTG TGC ACA TAC
 61  T   K   E   P   V   Y   K   S   P   F   S   V   V   N   Q   H   V   C   T   Y

241  GGC AAC TTC CGC TAT GAG ACG ATT CGT CTG CCT GAC TGT GCT GAT GGG GTG GAC CCT TTG  3
 81  G   N   F   R   Y   E   T   I   R   L   P   D   C   A   D   G   V   D   P   L

301  GTG ACC TAT CCA GTG GCC CTG AGC TGT GAG TGT AGT TTG TGC TCC ATG GAT ACG TCT GAC
101  V   T   Y   P   V   A   L   S   C   E   C   S   L   C   S   M   D   T   S   D

361  TGC ACC ATC GAG AGT GTG GAA CCC GAC TTC TGC ATG AGT CAG AGA CTG CCT GTT TAT GAA
121  C   T   I   E   S   V   E   P   D   F   C   M   S   Q   R   L   P   V   Y   E

421  AGT CAG AAA CCG TCT CTC TAT GAC TAT TAG cgttctgttctctcaataaatggatctcaac-Poly A
141  S   Q   K   P   S   L   Y   D   Y   *                                      149
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Sequenase 2.0 (US Biochemical Corp.). Sequencing was carried out using M13 Universal and Reverse primers. Sequencing gels of 6% polyacrylamide/7 M urea were dried and exposed to Kodak XAR-5 film for 24 h. Polypeptide sequences for GtH II-β (without the signal peptide) were analysed using the branch and bound option in PAUP (version 3.1.1 Swofford, 1993), and rooted with human LH.

RESULTS AND DISCUSSION

Nucleotide sequence of a herring pituitary cDNA encodes GtH II-β

A herring pituitary cDNA library was screened at low stringency with a probe corresponding to amino acids (aa) 36 to 108 of the open reading frame (Fig. 37). The complete sequence of 527 base pairs (bp) for herring GTH II-β cDNA is shown in Figure 37 (GenBank accession No. X91984 CHGTHIIB). The open reading frame of the herring GTH II-β cDNA sequence is 149 aa encoded by 450 bp including the start (ATG) and stop (TAG) codons. The 5'untranslated region (UTR) was 46 bp and and 3' UTR of 31 bp contained one putative polyadenylation signal AATAAA.

The putative signal peptide begins with a Met and is 24 aa in length as shown in Fig. 37. There is another potential translational start site 7 codons upstream, but the start site shown in Fig. 37 has a better context for initiation of translation because an adenine (A) is in the -3 position and a guanine (G) is in the +4 position (Kozak, 1991). Both features are missing in the vicinity of the upstream ATG codon. A hydrophobic core of isoleucine and leucines (ILLLL) is present in the center of the proposed signal peptide, as is typical of such peptides (von Heijne, 1983).

The putative cleavage site between the signal peptide and the herring GtH II-β subunit fulfills the rule that the amino acids at positions -1 and -3 of the signal peptide (relative to the cut site) are restricted to those with relatively small side chains (von Heijne, 1983). Alanine in the -3 and cysteine in the -1 positions in the herring GtH II-β signal peptide have also been reported in these positions in other signal peptides (von Heijne, 1983). Other potential cleavage sites in the herring carboxy region of the signal peptide were rejected because the residues did not conform to the -3 and -1 rule.

Conserved cysteine residues indicate that the isolated herring cDNA encodes a GtH II-β.

A comparison of GtH II-β polypeptides among herring and other fish is shown in Fig. 38. The signal peptides are included for species in which the cDNA of the GtH II-β has been determined. The mature herring polypeptide retains several features of other GtH II-β subunits. Chief among these features is the strict alignment of the 12 conserved cysteines. In contrast, the GtH a subunit contains only 10 cysteines and the GtH I-β has one of its 12 cysteines in a different position compared to GtH II-β. The GtH II-β cysteines are necessary to establish disulfide bonds with the a subunit and to determine tertiary structure essential for receptor binding (Quérat, 1994). Other conserved features that are thought to be important in binding to the a subunit and are shared by herring and other fish GtH II-β subunits include: a glutamic acid (E) four positions before the second cysteine, a Pro-Val-Ala (PVA) sequence before cysteine 8 and a teleost-specific Cys-Ser-Gly-His (CSGH) aa sequence (positions 31 to 34) between the fourth and fifth cysteine.

Fig. 38 Comparison of aa sequence of herring GTH II-β precursor with those of GTH II-β precursors or mature molecules of other teleosts. The aa sequences of the signal peptides and some mature GtH II-β molecules were derived from their corresponding cDNAs. The primary structures that were obtained by protein sequencing (in some cases in addition to nucleotide sequencing) are indicated by an * at the beginning of the sequence. The sequences are from Pacific herring (*Clupea harengus pallasi*; this paper), common carp (*Cyprinus carpio*; Jolles et al., 1977; Chang et al., 1988), silver carp (*Hypophthalmichthys molitrix*; Chang et al., 1990), catfish (*Clarias gariepinus*; Koide et al., 1992), pike eel (*Muraenesox cinereus*; Liu et al., 1989), European eel (*Anguilla anguilla*; Quérat et al., 1990), Arctic cisco (*Coregonus autumnalis*; Trofimova and Belikov, 1994), chum salmon (*Oncorhynchus keta*; Sekine et al., 1989), masu salmon (*Oncorhynchus masou*; Kato et al., 1993), chinook (*Oncorhynchus tschawytscha*; Trinh et al., 1986; Xiong and Hew, 1991), killifish (*Fundulus heteroclitus*; Lin et al., 1992), yellowfin porgy (*Acanthopagrus latus*; Tsai and Yang, 1995), bonito (*Katsuwonus pelamis*; Koide et al., 1993). The percent (%) identity of aa, not including those of the signal peptide, is shown compared to the herring molecule at the carboxy terminus of each hormone. Arrows indicate conserved cysteine residues.

GTH II-β ⊢ Signal Peptide ⊣

	1									50
HERRING	MARIPECTILLLLCMCVLAVPAQC	FNLQPCVLVN	ETVSVEKEGC	PRCLVFRTTI	CSGHCPTKEP	VYKSPFSVVN				
COMMON CARP	.GTPVKILVVRNHILFSVV.LLAVAQS	*SY.P..EP..	...A.....	.K...LQ...	.....L....	.....T.Y				
SILVER CARP	.LAVRNNIL..IF.LV..L.F..S	*SF.P..EP..	...A.....	.K....Q...	.....L....	.....T.				
CATFISH		*YL.TH.EP..	.....D..	.K..A.Q.S.	.....F....	.....SIY				
PIKE EEL		*SV....QPI.	..I....D..	.K....Q.S.	.....I..D.	S....L.T.Y				
EUROPEAN EEL	.SVY....W..FV.L.H.L.S.GG	SL.L..EPI.	..I....D..	.K....Q.S.	.....I..D.	S....L.T.Y				
ARCTIC CISCO	.LGLHVG.LMIS.FL.I.LE.VEG	SLM...QPI.	Q...L.....	.T...IQ.P.	.....F...L	.F.....T.Y				
CHUM SALMON	.LGLHVG.-.IS.FL.I.LE.VEG	*SLM...QPI.	Q...L.....	.T...IQ.P.	.....V....	.F.....T.Y				
MASU SALMON	.LGLHVG.-.IS.SL.I.LE.VEG	SLM...QPI.	Q...L.....	.T...IQ.P.	.....I....	.FR....T.Y				
CHINOOK	.LGLHVG.-.IS.FL.I.LE.IEG	SLM...QPI.	Q...L.....	.T...I.AP.	.....V....	.F.....T.Y				
KILLIFISH	.VCL---FLGASSFIWS..PA.AA	.Q.PR.Q.L.	Q.I.L..R..	SG.HRVE...	...Y.A..D.	N..TSYNKAI				
PORGY	.LPFMLSSF.GASPSIWPLA..EA	.Q.P..Q.I.	Q...L.....	.K.HPVE...	.....I..D.	.M.TRY--.Y				
BONITO		*.Q.P..Q.I.	Q.....	AT.HPVE...	.....I..D.	.I.I...K.Y				

	51									125	
HERRING	QHVCTYGNFR	YETIRLPDCA	DGVDPLVTYP	VALSCECSLC	SMDTSDCTIE	SVEPDFCMSQ	RLPVYESQKP	SLYDY	100%
COMMON CARP	......RDV.	...V.....P	P....HI...	.....D....	T.....	.LQ.....	.EDFLVY		74
SILVER CARP	......RDV.	...V.....P	P....HI...	.....D....	T.....	.LQ..Y....	.EDFPVY		74
CATFISH	......RDV.	.....R	P....H....	.....	I.....	.LN.....T.	KEFILDY		73
PIKE EEL	.R....RDV.	...V.....R	P....H..F.	.....D.N..	T.....A.Q	.LR.....	.A.		71
EUROPEAN EEL	.R....RDV.	...V.....R	P....H..F.	.....D.N..	T.....A.Q	.LR.....	.ASLPA		67
ARCTIC CISCO	......RDV.	....C....S	PW...H....	.....D....	N.....	.LQ..L..T.	.VLADG-		68
CHUM SALMON	......RDV.	.....P	PW...H....	.....D....	N.....	.LQ....IT.	.VLTDGDMW		69
MASU SALMON	......RDV.	..M.....P	PW...H....	.....D....	N.....	.LQ....IT.	.VLTDGDMW		67
CHINOOK	......RDV.	..M.....P	PWSE.H....	.....D....	N.....	.LQ....IT.	.VLTDGDMW		66
KILLIFISH	.....DLY	.K.FEF.E.V	P....V....	.....R.GG.	A.A.....F.	.LQ.....ND	IPFYH		66
PORGY	......RDLH	.K.FE....P	L....T....	..V..N.G..	A.....F.	.LQ.N...ND	IPFYY		62
BONITO	......RD.Y	.K.FE....P	P....I....	.....H.GR.	A.....F.	.LQ.....ND	IPFY.		55

The CSGH sequence occupies the equivalent position to the distinctive Cys-Ala-Gly-Tyr (CAGY) motif of mammalian LH-β (Quérat, 1994). Hence, Glycine (G), important for linkage to the a subunit, is present not only in the mammalian CAGY, but is also conserved in the teleost CSGH. A potential N-linked glycosylation site (Asn-X-Thr) is also present in positions 10 to 12 (NXT) of the herring molecule as in all other known GtH II-β molecules. These features clearly identify the herring sequence presented here as a GtH II-β.

Primary structure of herring GtH II-β has distinct features compared to those of other fish.

The difference in the deduced herring GtH II-β compared with other teleost GtH II-β polypeptides is primarily in the carboxy terminal region (aa 112 to 125). The additional amino acids at the carboxy end make the herring GtH II-β of 125 aa the longest one reported to date for fish. The carboxy termini are variable in other fish GtH II-β subunits as well and may indicate that this region is not functionally important. The rapid substitution rate in this portion of the molecule results in amino acid similarity only within closely-related groups: 1) the carps (common carp *Cyprinus carpio* L., silver carp *Hypophthalmichthys molitrix* Valenciennes) and eels (pike eel *Muraenesox cinereus* Forskal, European eel *Anguilla anguilla* L.), 2) the salmonid-like species (chum, *O. keta*; chinook, *O. tschawytscha* Walbaum; masu, *O. masou* Brevoort; Arctic cisco, *Coregonus autumnalis* Pallas) and 3) the more recently-evolved fish (killifish, *Fundulus heteroclitus* L.; yellowfin porgy, *Acanthopagrus latus* Houttuyn; and bonito, *Katsuwonus pelamis* L.). Herring has a carboxy terminus unrelated to the others.

Another region of difference in the protein sequence is the amino terminus. The consensus aa for the amino terminus is Ser (8/13 teleost species), but herring GtH II-β begins with phenylalanine (F) and hence matches the 3 advanced euteleost species rather than the more closely-related eels, carp and salmonids.

The overall aa sequence identity of the mature herring GtH II-β is closest to that of the carps, *C. carpio* and *H. molitrix* (74%), African catfish, *Clarias gariepinus* Burchell (73%), eels, *M. cinereus* and *A. anguilla* (67-71%), salmonids, *O. keta*, *O. tschawytscha*, *O. masou*, and *C. autumnalis* (66-69%) and killifish, *F. heteroclitus* (66%), and least to the more recent yellowfin porgy, *A. latus* (62%) and bonito, *K. pelamis* (55%). Whereas herring are most closely related to the salmonids in many characteristics (Lauder and Liem, 1983), this is not reflected in the similarity of the GtH molecule.

Phylogenetic analysis suggests a re-evaluation of the origin of GtH II -β Subunits

We constructed phylogenetic trees of mature β subunits of human, rat, chicken, and frog LH; human and frog FSH; fish GtH II from 16 species, including herring; and fish GtH I from two species. One most-parsimonious phylogenetic tree resulted, using a hueristic search (CI 0.799, rescaled CI 0.603, RI 0.754). Five principal clades with strong bootstrap support were found: carp, eels, salmonids, recently-evolved teleosts, and LH/FSH/GtH I (Fig. 39). Herring and catfish remain distinct from these clades. Identical principal clades were present if the PAUP analysis was carried out on the molecules without the 15 carboxy-terminal aa positions (relative to herring; results not shown), and in a neighbour joining (NJ) analysis (Fig. 40).

The phylogenetic trees show that the GtH II-β subunits of fish are similar amongst closely related species, but are otherwise quite diverse. Contrary to the hypothesis of a single early duplication and divergence of the GtH I and II genes (Quérat, 1994), our analysis indicates that LH, FSH and fish GtH I-β subunits are more closely related to each other than to other GtH II-β subunits. This pattern suggests that both the LH- and FSH-β subunits may have evolved from the fish GtH I-β lineage rather than that the LH subunit evolved from the GtH II-β lineage. However, the evolutionary distances calculated by the NJ analysis indicate that the divergence within the LH/FSH/GtH-I clade is considerably greater than in the clades of teleost GtH-II. More structures from phylogenetically ancient vertebrates are needed to elucidate the origins of these molecules. The apparent phylogeny of the GtH II-β subunit thus reflects accepted phylogenetic groupings of closely related teleosts, but shows unexpected divergences amongst teleost groups.

Fig. 39. A bootstrapped strict consensus phylogenetic tree of representative fish GtH II, GtH I, LH and FSH-β mature subunits, based on known amino acid sequences. The consensus tree is based on five most-parsimonious trees calculated with the branch-and-bound option of PAUP (Swofford, 1993). The numbers on branches of the tree indicate bootstrap likelihoods. Species and references for GTH II-β subunits not given in Fig. 38 are: sea bream (Sparus aurata; Elizur et al., 1996), striped bass (*Morone saxatilis*; Hassin et al., 1995), grouper (Elizur et al., 1996), chicken (*Gallus gallus*), frog (*Rana catesbeiana*). Sequences for human, chicken, and frog LH and FSH and chum and bonito GtH I-β subunits were used as given in Quérat (1994).

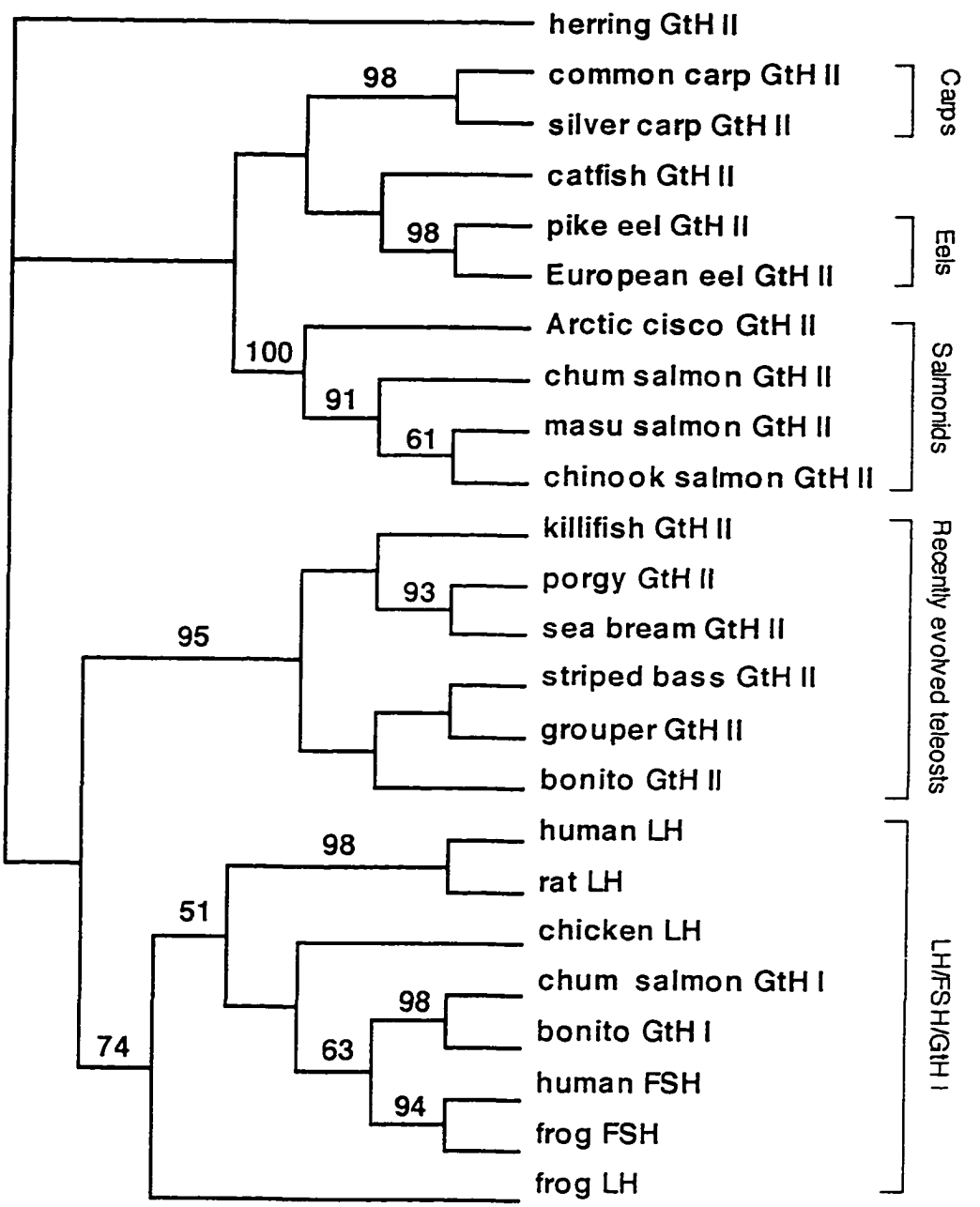
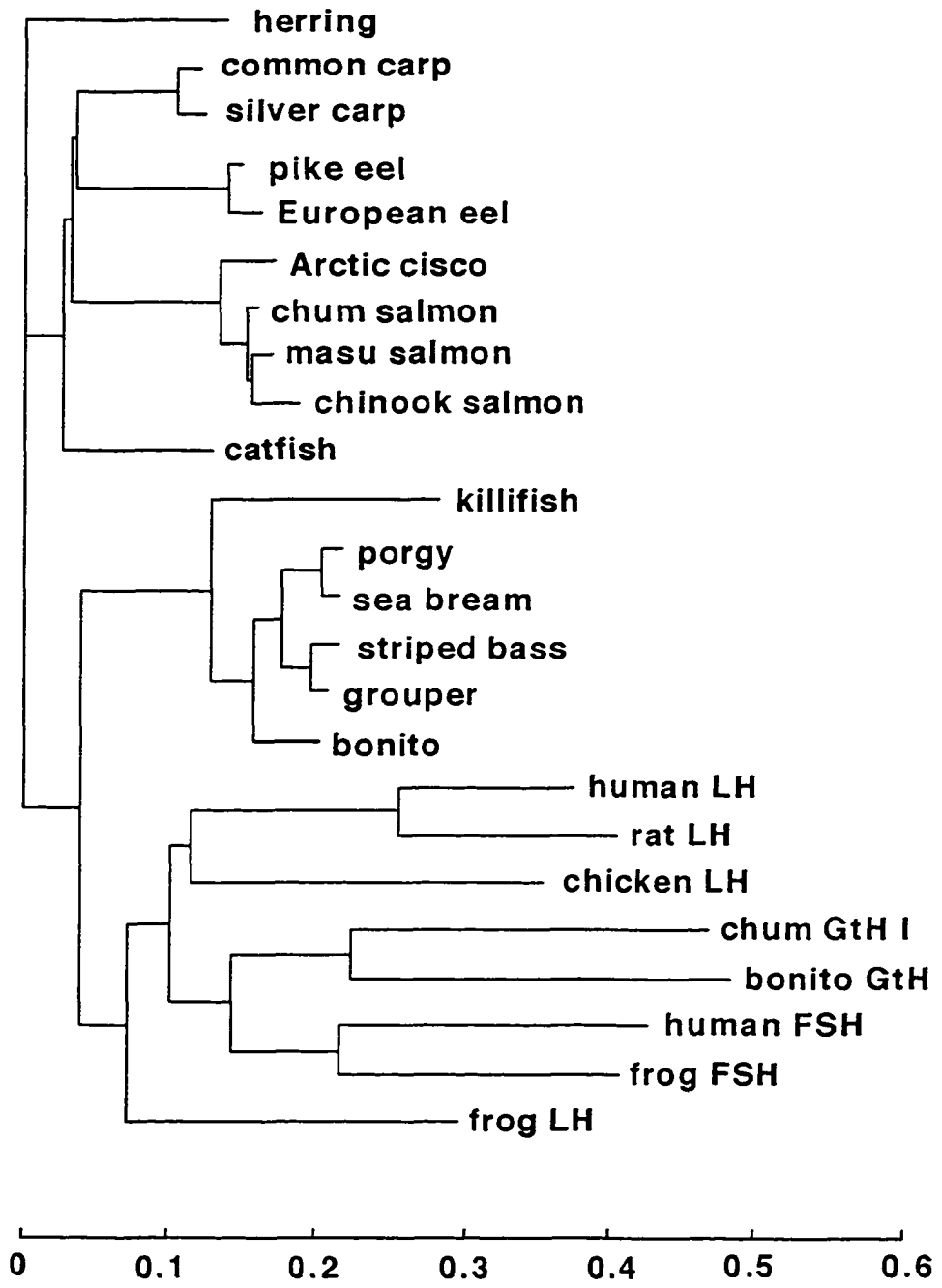

herring GtH II
98
common carp GtH II
silver carp GtH II
Carps
catfish GtH II
98
pike eel GtH II
European eel GtH II
Eels
Arctic cisco GtH II
100
chum salmon GtH II
91
masu salmon GtH II
61
chinook salmon GtH II
Salmonids
killifish GtH II
93
porgy GtH II
sea bream GtH II
95
striped bass GtH II
grouper GtH II
bonito GtH II
Recently evolved teleosts
human LH
98
rat LH
51
chicken LH
chum salmon GtH I
98
bonito GtH I
74
63
human FSH
94
frog FSH
frog LH
LH/FSH/GtH I

Fig. 40. Neighbour Joining (NJ) phylogram of mature vertebrate GtH-β subunits. Species names and references are as in Fig. 39. Distance measure used is the mean character difference (PAUP 4.0.0d47 software, D.L. Swofford, unpub.)


herring
common carp
silver carp
pike eel
European eel
Arctic cisco
chum salmon
masu salmon
chinook salmon
catfish
killifish
porgy
sea bream
striped bass
grouper
bonito
human LH
rat LH
chicken LH
chum GtH I
bonito GtH I
human FSH
frog FSH
frog LH
0
0.1
0.2
0.3
0.4
0.5
0.6

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