NPR

Discovery and characterization of natural products that act as pheromones in fish

Journal:	*Natural Product Reports*
Manuscript ID	NP-REV-01-2018-000003.R2
Article Type:	Review Article
Date Submitted by the Author:	29-Mar-2018
Complete List of Authors:	Li, Ke; Michigan State University, Buchinger, Tyler; Michigan State University , Department of Fisheries and Wildlife Li, Weiming ; Michigan State University

Natural Product Reports

ARTICLE

Discovery and characterization of natural products that act as pheromones in fish

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ke Li*, Tyler J. Buchinger, and Weiming Li*

Fish use a diverse collection of molecules to communicate with conspecifics. Since Karlson and Lüscher termed these molecules 'pheromones', chemists and biologists have joined efforts to characterize their structures and functions. In particular, the understanding of insect pheromones developed at a rapid pace, set, in part, by the use of bioassay-gudied fractionation and natural product chemistry. Research on vertebrate pheromones, however, has progressed more slowly. Initially, biologists characterized fish phermones by screening commercially available compounds suspected to act as pheromones based upon their physiological function. Such biology-driven screening has proven a productive approach to studing pheromones in fish. However, the many functions of fish pheromones and diverse metabolites that fish release make predicting pheromone identity difficult and neccesitate approaches led by chemistry. Indeed, the few cases in which pheromone identification was led by natural product chemistry indicated novel or otherwise unpredicted compounds act as pheromones. Here, we provide a brief review of the approaches to identifying pheromones, placing particular emphasis on the promise of using natural product chemistry together with assays of biological activity. Several case studies illustrate bioassay-guided fractionation as an approach to pheromone identification in fish and the unexpected diversity of pheromone structures discovered by natural product chemistry. With recent advances in natural product chemistry, bioassay-guided fractionation is likely to unveil an even broader collection of pheromone structures and enable research that spans across disciplines.

1. Introduction

Pheromones mediate aspects of physiology and behaviour in most animals. The chemistry and biology of insect pheromones are particularly well described, whereas research into the identity and function of vertebrate pheromones has progressed more slowly. Among vertebrates, fishes are especially reliant upon pheromones, and research into fish pheromones offers new tools to manage invasive species and restore imperilled species as well as general insights to the function and evolution animal communication. Many of the early strides in our understanding of fish pheromones came from biology alone. Biologists made predictions of pheromone identity based on the physiological function of known compounds and defined important concepts that helped shape the field. However, limited integration with natural product research left many biologically active substances undescribed. Recent collaborations between chemists and biologists enabled additional approaches to pheromone research that emphasize chemical characterization of biologically active compounds as opposed to targeted screening of known compounds. This approach led to unexpected insights; several of the pheromones identified have chemical properties not predicted based upon previous screening of known compounds.

Here, we review the literature at the intersection of natural product chemistry and pheromone communication in fish. Several excellent reviews on pheromones in fish and other taxa set the foundation for our discussion.[1-4] For the purposes of our review, we adhere to the original definition proposed by Karlson and Lüscher,[5] which states that pheromones are

Department of Fisheries and Wildlife, Michigan State University, Room 13 Natural Resources Building, 480 Wilson Rd., East Lansing, Michigan 48824, United States. E-mail: like4@msu.edu, Tel: +1-517-432-2778; liweim@msu.edu, Tel: +1-517-432-6705, Fax: +1-517-432-1699

"substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process". Although chemical ecologists have refined the definition with useful modifications,[6] we follow an inclusive definition to place emphasis on chemistry rather than the biological function. However, we exclude learnt or self-referent signature mixtures as identifying specific compounds is especially difficult and less likely to be generalizable across individuals.[6] After providing a brief overview of the function and identity of known fish pheromones, we review several approaches used to characterize pheromones and highlight case studies that exemplify the use of natural product methods in fish pheromone research.

1.1 Biological functions of fish pheromones

Living in an often visually-obscured environment, fishes have evolved to rely largely upon olfaction to collect information about their surroundings.[2] We define olfaction based upon the underlying mechanisms through which fish sense molecules; fish detect odorants using olfactory neurons that project via cranial nerve 1 to the olfactory bulb.[7] Importantly, animals likely detect most pheromones with the olfactory system.[6] Olfactory cues mediate social interactions associated with aggregation, reproduction, risk, kin recognition, and various other functions. Many insightful reviews cover the physiology, ecology, and evolution of olfactory cues and pheromones, and readers should consult these for in-depth discussions on pheromone biology.[1, 2] Although many studies describe behavioural responses of fish to conspecific odours, few report the effects of specific compounds, precluding definitive conclusions of the involvement of pheromones versus learnt or self-referent cues. One of several exceptions is the sea lamprey (*Petromyzon marinus*),[8] a jawless fish whose complex life cycle provides a useful example of the diverse functions of pheromones in fish (Fig. 1).

The sea lamprey is a jawless fish that uses pheromones to migrate into spawning streams, synchronize sexual maturation, find mates, and avoid risk.[8] During the migration from feeding habitat in lakes or the Atlantic Ocean, adult sea lamprey move into streams activated with the odour of stream-resident larvae[9-11] and avoid areas activated with dead conspecifics.[12-14] Once sexually mature, males and females release pheromones that mediate sexual maturation, mate search, and spawning behaviours in conspecifics.[9, 15-17] Much of the research on sea lamprey pheromones focuses on adult behaviour, but larvae also respond to the odour of dead conspecifics[18, 19] and adjust growth rates upon exposure to conspecific odours.[20] Taken together, the roles of pheromones across the life cycle of sea lamprey illustrate the diverse biological functions and chemical identities of pheromones in fish.

1.2 Chemical identities of fish pheromones

The fish olfactory system detects several types of compounds, some of which function as pheromones.[21] Although most pheromones in fish remain unidentified, the few that are structurally identified include a variety of low-molecular-weight metabolites, such as bile salts,[22-24] F-series prostaglandins,[25] amino acids,[26] and gonadal steroids.[27] Although many assume that solubility is critical for aquatic pheromones, recent discussions make an important point that solubility may not be required but rather determine the spatial range of a pheromone.[28, 29] Consistent with this point, many mating pheromones in fish are not readily or completely soluble in water.[29] Notably, most pheromones are mixtures of multiple compounds and no mixture has been completely elucidated in fish; hence, the structures discussed here are only components of pheromones. Furthermore, some compounds identified from a natural pheromone blend are potent odorants with no reported function. Continued research into fish pheromones is sure to unveil many more pheromone structures.

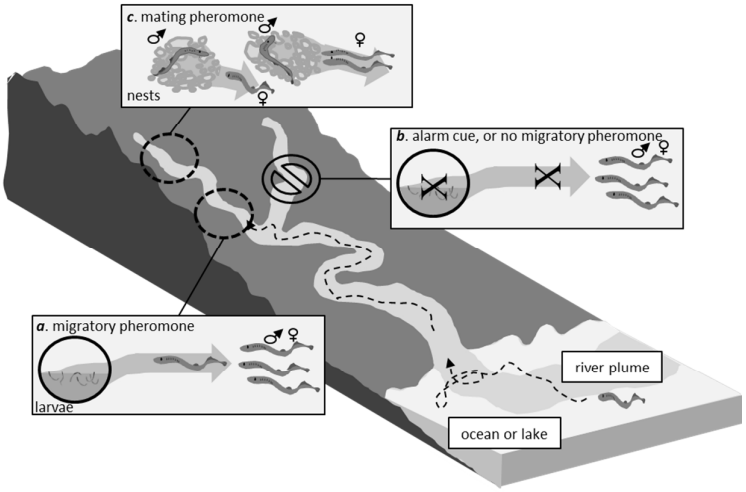


Fig. 1 Schematic illustrating the hypothesized functions of pheromones use by sea lamprey during reproduction. a) Fewer migrating sea lamprey enter rivers or tributaries with injured or decaying conspecifics, or lacking larval populations; b) Migrating sea lamprey enter streams activated with larval odourants; c) upon reaching sexual maturation males release a mating pheromone that draws females to spawning nests, and initiate nest building and spawning behaviours. Modified from Buchinger et al., 2015[8]

Sex steroids

Sex steroids are hormones produced from cholesterol primarily in gonads (ovaries or testes) or adrenal glands and modified in various tissues. Sex steroids have a basic structure of four carbon rings called the cyclopentanoperhydrophenanthrene ring or steroid nucleus. The structures of sex steroids include three major classes: androgens, estrogens, and progestogens. Androgens possess a 19-carbon androstane skeleton, estrogens an 18-carbon estrane skeleton, and progestogens a 21-carbon pregnane skeleton. Most modifications to this basic structure relate to the functional group on C-3 or C-17. For example, the estrogen derivatives estrone, estradiol, estriol, and estetrol vary by ketonization on C-17 and hydroxylation on C-3, C-17, C-16, and C-15 of its backbone.

Sex steroids and their derivatives act as reproductive pheromones in many teleost fishes.[30-32] Fishes use sex steroids as hormones that modulate various aspects of their sexual physiology and behaviour. Knowing this, biologists correctly predicted that aquatic animals, including fish, leak hormones into the water where they act as pheromones.[33, 34] Goldfish (*Carassius auratus*) offer an exquisitely comprehensive example of how sex hormones, namely sex steroids and prostaglandins, can guide inter- and intra-sexual behaviours during reproduction (briefly reviewed below).[32] Although hormonal pheromones likely evolved after fish passively leaked them into the water, several fish seem to alter hormone release using urinary pulses.[35-37] Conjugation of sex steroids with glucuronic acid and sulphates are also be important to their role as pheromones.[38] The discovery that many fishes use sex hormones as pheromones highlights the promise of using biology to identify behaviourally active compounds.

Prostaglandins

Prostaglandins are a diverse group of physiologically active lipids with 20 carbon atoms, including a 5-carbon ring. Synthesized from arachidonic acid, prostaglandins are a subclass of eicosanoids and of the prostanoid class of fatty acid derivatives. There are four principal series of prostaglandin structural backbones: E-series, I-series, D-series, and F-series. In addition, the side chains can have one, two, or three double bonds, and the five-carbon ring can have double bonds, a ketone, or alcohol groups. Prostaglandins were first isolated from seminal fluid [39, 40] and described in the context of mammalian reproduction,[41] but are now known to occur in most tissues and have a wide range of functions.[17] Notably, females of some fishes use F-series prostaglandins as hormones that trigger sexual behaviour and leak prostaglandins into the water immediately after ovulation.[25] Similar to sex steroids, F-series prostaglandins released into the water act as pheromones in goldfish and likely many other fishes.[32, 42]

Bile salts

Bile salts are structurally and functionally diverse steroids derived from cholesterol in vertebrates. The structural diversity of bile salts follows a systematic pattern of variation across vertebrates, and includes the generic structures of 27-carbon bile alcohols, 27-carbon bile acids, and 24-carbon bile acids, each with default hydroxylation at C-3, C-7 or/and C-12.[43, 44] The 27-carbon bile alcohols predominate in lampreys, hagfish, cartilaginous fishes and amphibians, and 27-carbon bile acids in reptiles and early evolving birds. The 24-carbon bile acids are present in all vertebrate classes, often with 27-carbon alcohols or 27-carbon acids, indicating two evolutionary pathways from 27-carbon bile alcohols to 24-carbon bile acids. The bile salts produced by fishes are especially diverse, and vary in side chain functional groups and length, A/B ring juncture, skeleton and side chain hydroxylation and ketonization, and conjugation.[43, 44] Notably, invertebrates generally do not possess bile salts. The major function of bile salts is solubilisation of fats during digestion, but additional functions include cholesterol homeostasis, endocrine signalling, and antimicrobial actions.[45, 46] Given their functional diversity and systematic variation across vertebrates, bile salts might serve as omnipresent odourants in aquatic environments that confer taxon-specific information.

Bile salts are potent odourants for many fishes.[47] Døving *et al.* first reported the olfactory sensitivity of fishes to bile salts, and suggested they may guide the migration of Arctic char (*Salvelinus alpinus*) and other salmonids into spawning streams.[48] Additional studies revealed that bile salts stimulate the olfactory systems of many fishes, and potentially guide various behaviours in addition to migration. Despite widespread evidence that fishes detect bile salts, biologists understand very little about their ecological function as odorants, except in sea lamprey which follow conspecific bile acids, together with unknown compounds, when searching for spawning streams and mates.[24, 49-51] Notably, some bile acids (*i.e.* tauocholic acid) are released by many species and unlikely species specific.[47] In contrast, others are taxa-specific and released by offspring as metabolic waste, and therefore fitting candidates to be migratory pheromones that guide adults searching for productive spawning streams.[49] However, the role of the bile acid sex pheromone in sea lamprey,[24] discovered through bioassay-guided fractionation, was less expected and underscores the unpredictability of nature.

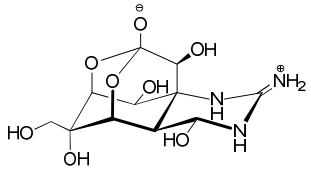
Amino acids

Amino acids are ubiquitous compounds with amine, carboxylic acid, and side-chain groups and are at the core of many biochemical processes. The diversity of amino acids can be categorized by additional functional groups, such as the basic amino acids possessing an amine group, acidic amino acids with a carboxylic acid group, and neutral amino acids with hydroxyl and sulfhydryl groups. Amino acids also have chiral center(s) that could add to their diversity although most that occur in nature are $_L$-amino acids.

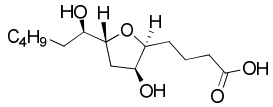
Fishes detect amino acids with their olfactory system,[17] potentially to mediate behaviours associated with feeding,[21, 52, 53] dominance hierarchies,[54] migration,[55] and reproduction.[26, 56] For example, salmon may learn amino acid profiles of their natal stream and use the odour to return to spawn as adults.[55] However, chemical ecologists generally expect amino acids to function as general cues not pheromones given their widespread occurrence and hence low specificity.[57, 58] Contrary to this expectation, bioassay-guided fractionation of urine from masu salmon (*Oncorhynchus masou*) led to the discovery that the amino acid L-kynurenine is a sex pheromone,[56] again highlighting the surprising results of studying pheromones as natural products.

Others

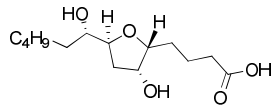
Identified fish pheromones are not limited to sex steroids, prostaglandins, bile salts, and amino acids. For example, tetrodotoxin (TTX, **1**) is a potent neurotoxin that female grass pufferfish (*Fugu niphobles*) release with ovulated eggs into the water, where it attracts sexually mature males.[59] Likewise, several tetrahydrofuran diols [petromyroxols (**2**-**3**) and *iso*-petromyroxols (**4**)] are potent odourants released by larval sea lamprey, indicating fatty acids may also function as pheromones. These results underscore the importance of studying pheromones without strong predispositions to the potential chemical structures involved. Additional classes of compounds are likely to emerge as pheromones in the future given most pheromones in fish remain unidentified and many types of responses orphaned from any particular compound.



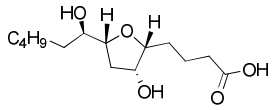
tetrodotoxin (**1**)



(+)-petromyroxol (**2**)



(–)-petromyroxol (**3**)



iso-petromyroxols (**4**)
enantiomer is arbitrarily shown

2. Approaches to pheromone identification

Biologists and chemists characterize pheromones using various approaches and degrees of integration. The requisite first step is observing a behavioural or physiological reaction to a conspecific odour. In efforts to identify fish pheromones, biologists often rely on electro-olfactogram recordings to determine whether a compound stimulates a fish's olfactory epithelium and hormonal or behavioural assays to determine if odourous compounds elicit the original reaction to the natural pheromone. Subsequent to observing a response, researchers might screen commercial compounds with physiological functions somehow linked to the focal reaction (targeted screening of known compounds), profile the metabolites in the odour (metabolomics), or identify the active compounds using natural product chemistry together with biological assays (bioactivity-guided fractionation). Field tests complement each method, as laboratory results are not always replicable in natural environments.[60] Notably, targeted screening and bioactivity-guided fractionation currently predominate the fish pheromone literature, but other approaches such as metabolomics also hold much promise.[61]

2.1 Targeted screening of known compounds

Biologists have correctly predicted the likely structure of several pheromones based upon the physiological function of a compound. Fish release an array of metabolic by-products including hormones, respiratory and digestive waste, and compounds otherwise associated with internal physiological functions. The prevailing model of pheromone evolution predicts that animals evolve responses to metabolic by-products released into the water for reasons unrelated to communication.[33, 34, 62] Knowledge of the metabolic by-products of particular physiological processes allows biologists to hypothesize the likely structure of pheromones that guide interactions among conspecific individuals in certain contexts. The identification of sex hormones as important reproductive pheromones exemplifies the logic of targeted screening, whereas fish leak sex hormones during specific points in their reproductive cycle and these leaked hormones indicate a fish's reproductive status to other fish. Commercial availability of many sex hormones allows biologists to screen compounds for bioactivity once identified as potential pheromones. In addition to predicting sex hormones act as reproductive pheromones, biologists also predicted that bile salts released during digestion might guide aggregation in productive habitat.[48, 63] Targeted screening has proven useful in characterizing several pheromones, but left many unidentified because biologists can only use it to test known and available compounds.

2.2 Metabolomics

Metabolomics is the global quantitative assessment of endogenous metabolites within a biological system. Chemists use several platforms to profile metabolites, including nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS). Comparing two or more metabolomes can reveal components that distinguish groups and is particularly advantageous by allowing high-throughput chemical profiling with no required purification steps. Though a relatively young field, metabolomics is already applied extensively to disease diagnoses,[64] environment analyses,[65] and toxicology,[66] as well as several aspects of fish biology.[67] Unlike biology-driven screening or bioassay-guided fractionation, metabolomics usually employs multivariate analyses, which enable identification of multiple components

that may act together but elicit no response independently. However, the availability of synthetic references constrains pheromone identification using metabolomics, as the resulting data do not implicate specific chemical structures. To our knowledge, no one has used metabolomics to characterize pheromones in fish, but research on other organisms indicates that it is a useful approach to consider when studying chemical ecology in any taxa.[61, 68, 69]

2.3 Bioassay-guided fractionation

Bioassay-guided fractionation integrates biology and natural product chemistry to identify pheromones (Fig. 2). This approach has been an impetus for identification of a large number of pheromones from insect and other animals. However, few researchers use bioassay-guided fractionation to identify pheromones in fish, likely because isolation and structural elucidation of the larger water-soluble pheromones used by fish is more challenging than for the small volatile pheromones used by insects. Nonetheless, the few studies that used bioassay-guided fractionation with fish yielded unexpected pheromone identities, highlighting the reward of the approach despite its challenges.

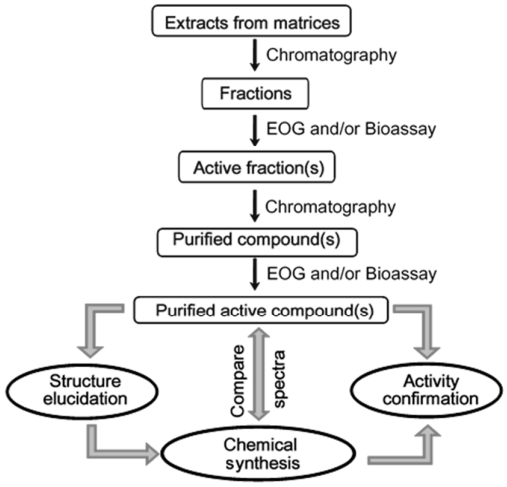


Fig. 2 Steps for isolation and identification of pheromone component(s). See Fine and Sorensen 2008 for specific example.[70]

The process of bioassay-guided fractionation begins with collecting large quantities of natural pheromone. Enrichment and extraction are especially critical because fish generally do not have pheromone-producing glands and, as a result, samples often come from the water surrounding a fish. Like most studied animals, fish usually release pheromones in minute quantities and under specific conditions. Most researchers use one of several types of solid phase extraction (SPE) to extract fish pheromones. Initially, many used silica-based reversed phase (C18) SPE cartridges, but less hydrophobic reversed phase sorbents (i.e., C8, C3, CN, and phenyl) are usually more appropriate for polar molecules. However, issues associated with using reversed phase SPE include sample loss by irreversible adsorption of basic compounds, hydrolysis of the silica backbone in high pH environments, and intensive labour required to avoid sorbent drying.[71] To circumvent such problems, environmental chemists are increasingly using sorbents with a polystyrene and/or divinyl benzene backbone such as Oasis® HLB (Waters Corporation) or Strata™-X (Phenomenex), especially when concentrating highly polar chemicals such as pharmaceuticals and pesticides. These sorbents have a higher capacity, tolerate partial drying, and are effective over a wide pH range. Indeed, the aforementioned sorbent SPE methods are impractical for large-scale sampling. Chemical ecologists studying fish usually use SPE in columns, although Stewart and Baker describe a method that uses passive sampling of pheromones.[72] Overall, the objective is to capture the biological activity from the original sample while also collecting a large quantity of compounds to pass through fractionation and bioassays.

Bioassay-guided fractionation is a standard method to hone in on the active compound(s). After confirming the extraction method successfully captures all bioactivity, chemists use chromatographic methods such as size exclusion chromatography (i.e., gel filtration), reversed phase chromatography, and affinity chromatography to separate groups of compounds and, eventually, individual compounds. Between each separation step, biologists use physiological or behavioural assays to determine which fractions or isolated compounds elicit biological responses. Notably, individual components of multi-component pheromones may separate into different fractions; hence, tracking the bioactivity of pheromone mixtures that function synergistically can be a major challenge. After the extract is narrowed down to active fractions and components, isolated compounds are advanced through several techniques for structural elucidation.

The diversity of potential pheromones in fish demands particular emphasis on structural elucidation of compounds yielded by bioassay-guided fractionation. As highlighted in the case studies below, fish use diverse and often novel compounds as pheromones, complicating predictions of pheromone identity based on function or comparisons to standards. Natural product chemists elucidate the structure of bioactive compounds using mass spectroscopy, nuclear magnetic resonance (NMR), or X-ray crystallography. Most fish and insect pheromones have unpredictable stereochemistry; hence, chemists determine the relative configuration of bioactive molecules using NOESY and ROESY and the absolute configuration using analogue comparisons, derivative reactions, and X-ray crystallography. Identified structures of bioactive compounds released by fish are then synthesized for further characterization.

Synthesizing the product of bioassay-guided fractionation allows structural confirmation and biological characterization. Comparing chemical properties such as optical rotation, melt points, molecular weight, and proton and carbon resonances between the purified and synthesized compounds confirms the proposed structure. Bioassays then determine whether the synthetic compound elicits the appropriate responses in laboratory assays. Given responses in the laboratory do not always translate to responses in the field,[60] synthesis of

putative pheromones is critical by providing the large quantity of compound needed for field tests. Synthesized compound also provides the standard required to quantify pheromone released by fish into the environment, which, together with field studies, allows for a comprehensive biological characterization of a pheromone. Lastly, synthesizing structural analogues of identified pheromones can allow studies that unveil structure-function relationships and potentially new, structurally similar pheromones.[73]

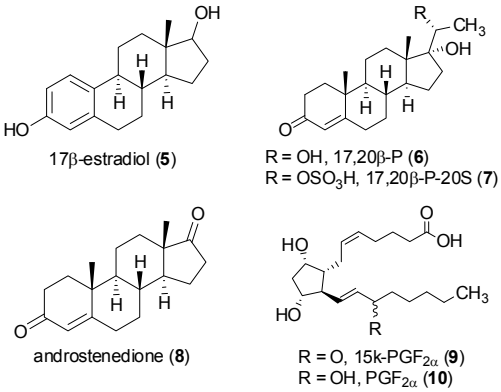
3. Case studies

3.1 Sex hormones synchronize spawning in goldfish

Fig.3 Goldfish (*Carassius auratus*, Wikipedia)

The goldfish (Fig. 3) is an important model that illustrates the utility of targeted screening for identifying pheromones in fish. Research on goldfish, one of the first described and now best understood models with regard to fish pheromone communication, laid the foundation for pheromone studies in many other fish and defined key concepts in the field. Several reviews discuss the hormones and pheromones involved in goldfish reproduction;[32, 74, 75] hence, we provide only a selective overview the goldfish pheromones identified using biology-driven screening.

Goldfish use a suite of hormones and hormone metabolites as pheromones during reproduction.[32, 74, 75] Research to identify goldfish pheromones built off observations that females release three distinct odours in the weeks, hours, and minutes before spawning. Males also release a pheromone but we focus here on female pheromones, which are better understood. Biologists first turned to the endocrine control of fish reproduction after noting the close link between the release and function of each pheromone and the internal reproductive events of vitellogenesis, the end of vitellogenesis, and ovulation. Plasma levels of 17β-estradiol (**5**) peak during vitellogenesis but olfactory screening indicated males do not detect 17β-estradiol or its common metabolites as odourants, leaving the pheromone released weeks before spawning unidentified. Upon completing vitellogenesis, females secrete various sex steroids associated with final oocyte maturation. Targeted screening of these steroids indicated a portion of them stimulate olfactory epithelia in goldfish, and physiological and behavioural assays indicated 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P, **6**), 17,20β-P-20S (**7**), and androstenedione (AD, **8**) elicit responses in sexually mature males. After ovulation, the final reproductive stage for females, goldfish synthesize various prostaglandin metabolites, which act on the brain to initiate female sexual behaviour. Olfactory and behavioural screening of several prostaglandin metabolites that females release into the water indicated 15keto-prostaglandin $F_{2\alpha}$ (15k-$PGF_{2\alpha}$, **9**) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, **10**) elicit sexual behaviour in males.[32, 74, 75] Taken together, research on goldfish exemplifies the promise of predicting pheromone identity based upon physiological and behavioural function, but also highlights the need for other methods when pheromones are novel or unexpected compounds.



3.2 Sulphated bile acids guide reproduction in sea lamprey

Fig. 4 Sea lamprey (*Petromyzon marinus*)

Sea lamprey (Fig. 4) use a diverse set of molecules as pheromones during reproduction. As the first fish species in which bioassay-guided fractionation unveiled pheromone identities, sea lamprey epitomize the benefits of integrating untargeted natural product chemistry and bioassays to identify pheromones. Most of the pheromone compounds were novel, in some cases with novel skeletons, and unpredicted given their behavioural functions to guide migration and spawning.

Migratory sea lamprey adults select spawning habitat following a pheromone released by stream-resident larvae. Initially, the approach to identify the sea lamprey migratory pheromone mirrored biology-driven screening, whereas biologists predicted bile acids released by stream-resident juveniles indicate productive habitat to adults searching for

spawning sites.[22] Support for the hypothesis came from evidence that known lamprey bile acids stimulate the olfactory epithelium of sea lamprey[49] and influence their behaviour in laboratory assays.[50] However, the known bile acids did not elicit the complete response to larval odour, necessitating bioassay-guided fractionation to elucidate the unknown components.

Bioassay-guided fractionation implicated unexpected and novel bile acids as potential migratory pheromones in sea lamprey.[76] To start the process, an intensive extraction using Amberlite XAD7HP resin collected the large quantity of pheromone needed to proceed through several iterations of fractionation and bioassays.[76] For example, extraction of 8000 litres of water containing ~35 000 larvae yielded ~200-1000 µg of putative pheromone compounds.[76] Increasingly discriminate fractionation guided by bioassays and ESI-MS techniques indicated three compounds elicit behavioural responses similar to those to larval odour in laboratory mazes. Notably, synergism between the larval odour and natural stream odour required that behavioural assays be conducted with stream water.[10] After purification using reversed phase HPLC, MS and NMR indicated the pheromone consisted of two novel compounds petromyzonamine disulfate (PADS, **11**), petromyzosterol disulfate (PSDS, **12**), [33, 84] and the previously known sea lamprey bile acid derivative 5α -petromyzonol-24-sulfate (3α,7α,12α,24-tetrahydroxy-5α-cholan-24-sulfate, PZS, **13**).[50] Synthetic chemistry allowed close examination of the configurations at the C-3 and C-24 of PADS and PSDS and provided unambiguous evidence for a tetracyclic framework and steroid side chain.[77] Not only were these compounds interesting in that they were impossible to predict, but their structures were also broadly interesting as natural products. For example, PADS possesses a rare N-(3-aminopropyl) pyrrolidin-2-one subunit that may originate through oxidation of squalamine (**14**), an antimicrobial compound isolated from the dogfish shark.[78, 79] Despite the evidence that PADS, PSDS, and PZS contribute to the biological activity of larval odour in a maze, field tests of the individual compounds and their mixture indicated additional components remain unidentified.[80, 81]

Additional rounds of bioassay-guided fractionation of larval odour implicated a sulphated hexahydrophenanthrene and four fatty acid enantiomers as putative pheromones. First, Li et al. isolated and characterized petromyzonin (**15**)[82] and determined its absolute configuration using electronic circular dichroism (ECD) spectroscopy. Interestingly, petromyzonin, possibly derived from estrone, represents a novel carbon skeleton and the first example of a sulphated hexahydrophenanthrene. Second, bioassay-guided fractionation led to the isolation and characterization of four novel tetrahydrofuran (THF)-diol fatty acid enantiomers, named (+)- and (−)-petromyroxol (**2** and **3**, respectively),[83] (+)- and (−)-iso-petromyroxol (**4**).[84] Mosher reaction techniques[85, 86] elucidated the absolute configurations of (+)- and (−)-petromyroxol, while (+)- and (−)-iso-petromyroxol were at insufficient quantities to determine their configurations. A plethora of natural products with various biological functions possess a THF ring[87], but none had been shown to act as pheromones. Notably, the petromyroxols also provide an interesting template system for synthetic chemists.[88-92] Petromyzonin (**14**) and (+)-petromyroxol (**3**) each stimulate the lamprey olfactory system and may function as putative migratory pheromones. Taken together, several rounds of bioassay-guided fractionation implicated a diverse collection of novel and unpredicted molecules as putative pheromones.

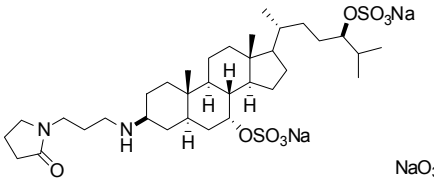
The mating pheromone released by male sea lamprey comprises a collection of bile acids unexpected given its sexual function. As discussed above, many fish appear to use sex hormones as reproductive pheromones, presumably due to the close association between the reproductive status of a fish and the hormones they release into the water. Sea lamprey also detect several sex steroids[93] and biologists once considered them candidate pheromones to guide mate search. However, bioassay-guided fractionation led to the unexpected discovery that a major component of the male pheromone is a lamprey-specific 5α, C24 bile acid known as 3keto petromyzonol sulfate (3kPZS, **16**).[24] Interestingly, the male pheromone consists of multiple components and 3kPZS likely facilitates long-distance mate search while minor components elicit near-source courtship behaviours.[16] Continued fractionation of the male pheromone yielded several additional steroids named petromyzestrosterol (**17**), 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES, **18**),[94] petromyzene A (**19**) and B (**20**),[95] and petromyzone A–C (**21**-**23**).[96] Each has all *trans* conjugated rings and a 5*α*-H, except petromyzene A and B, petromyzone C, and DkPES which lack the 5*α*-H due to a ketene substitution on C-3-C-6. Petromyzene A features a rearranged side chain unique among C-24 bile salts, while petromyzene B possesses a rare *cis*-11,12-diol on the steroidal C-ring. Further analysis of water conditioned with mature male sea lampreys indicated the presence of four additional oxidized, unsaturated compounds. Mass spectrometry indicated four unsaturated sulfated bile alcohols tentatively identified as 12-keto-1,4-diene 3kPZS (**24**), 12-keto-4-ene 3kPZS (**25**), 4-ene 3kPZS (**26**), and 1-ene 3kPZS (27).[97] Although several of these compounds elicit olfactory and behavioural responses in females, understanding their role as minor components of the male pheromone requires additional experiments. Consistent with the hypothesis that aquatic cues need not be water soluble,[29] among all the putative pheromones isolated from sea lamprey, only 3kPZS is water soluble according to the values estimated with WSKOWIN™ v1.42 (for water solubility) and MPBPWIN™ v1.43 (for vapour pressure) programs from EPI Suite™. Not only are these bile acids novel, but their potential roles as mating pheromones are surprising given there is no direct link between bile acid release and a fish's reproductive status, and no other fish are known to use bile acids as mating pheromones.[47]

Based upon research on mammals [98, 99], we propose a pathway through which sea lamprey biosynthesize bile salt pheromones from cholesterol. Elliot and Hyde [100] suggest animals synthesize 5α intermediates via cholestenol. To form a C-27 sulphated bile salt skeleton, a 24-hydroxylase[99] and C-24

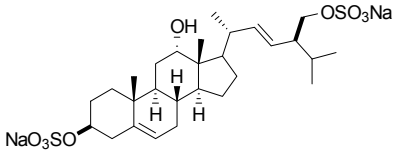
half sulphur ester conjugate to form an intermediate that is further condensed with *N*-(3-aminopropyl)pyrrolidin-2-one and sulphated to form PADS (**11**) , and hydroxylated on C-11 and C-12 to yield petromyzone A (**21**).[96] 3kPZS (**16**), a C-24 sulfated bile alcohol, likely forms after side-chain cleavage by five liver peroxisomes and conjugation with a half sulfate ester, although PZS may also be oxidized to form 3kPZS.[101, 102] 3kPZS can be considered as the "template" for other C24 bile salts in the sea lamprey bile salt profile. Additional modifications

Natural Product Reports

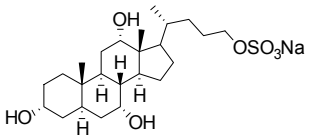
ARTICLE



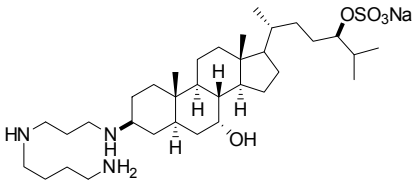
petromyzonamine dimonosulfate (PADS,**11**)



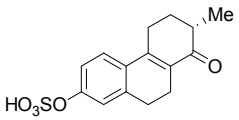
petromyzosterol disulfate (PSDS, **12**)



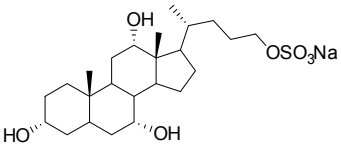
petromyzonol sulfate (PZS, **13**)



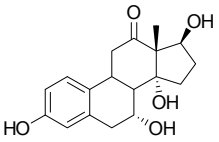
squalamine (**14**)



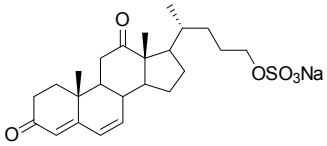
petromyzonin (**15**)



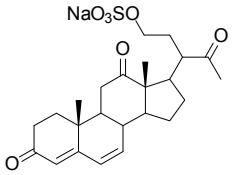
3kPZS (**16**)



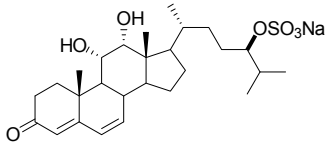
petromyzestrosterol (**17**)



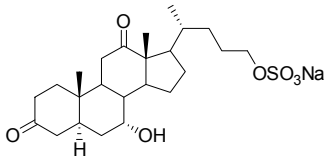
DkPES (**18**)



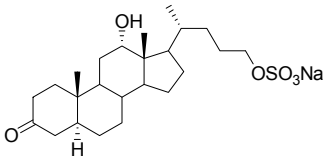
petromyzene A (**19**)



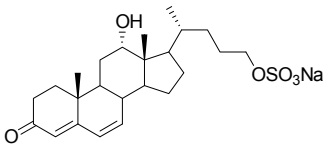
petromyzene B (**20**)



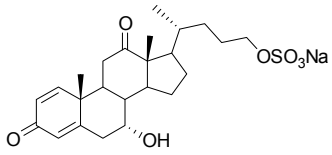
petromyzone A (**21**)



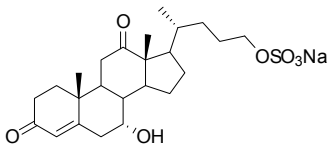
petromyzone B (**22**)



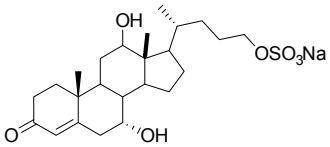
petromyzone C (**23**)



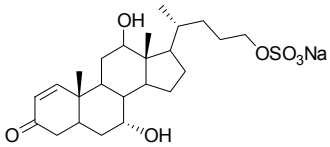
12-keto-1,4-diene 3kPZS (**24**)



12-keto-4-ene 3kPZS (**25**)



4-ene 3kPZS (**26**)



1-ene 3kPZS (**27**)

Natural Product Reports

ARTICLE

(hydroxylation, double bonds) to 3kPZS account for the characterized derivatives, such as petromyzone B,[96] DkPES,[103] and PZS.[104]

Fig. 5 Mozambique Tilapia (*Oreochromis mossambicus,* Wikipedia)

3.3 Steroid epimers prime female tilapia for reproduction

Mozambique Tilapia (*Oreochromis mossambicus*, Fig. 5) use urinary pheromones to evaluate a female's reproductive status and a male's dominance status.[105] Olfactory screening of commercially available sex steroids indicates that, as in goldfish, female-released sex steroids are likely candidates of the female pheromone.[105, 106] Biologists applied the same logic in efforts to identify male pheromones, but found that most commercially available sex steroids elicited only trivial olfactory responses in Mozambique Tilapia.[107, 108] Subsequently, bioassay-guided fractionation of dominant male urine led to the identification of the first male pheromone in a teleost fish.[105]

A pair of novel pregnanetriol 3α-glucuronate epimers in dominant male urine stimulates the endocrine system of female Mozambique Tilapia (Fig. 6).[107] Females exposed to male urine increase release of the oocyte-maturation hormone 17,20β-P.[106] Guided by hormone secretion assays, initial fractionation indicated that the active component in male urine was a sulphated amino-sterol-like compound with a formula of $C_{29}H_{40}N_2O_{10}S$.[109] Continued fractionation used HPLC with evaporative light scattering detection (ELSD) to allow observation of a signal despite the absence of chromophores. UPLC-HR-MS revealed the presence of two compounds with similar mass to charge ratios and retention times (*m/z* 511.2908 and 511.2912, 12.78 and 12.82 min, respectively) and indicated both compounds had the same molecular formula. The molecular formula was $C_{27}H_{43}O_9$, in contrast to the previously proposed sulphated amino-sterol-like compound with a formula of $C_{29}H_{40}N_2O_{10}S$.[109] 2D NMR and comparisons with the analogues indicated the compounds were two stereoisomers of the steroid pregnanetriol 3-glucuronate, 5β-pregnane-3α,17α,20α-triol-3α-glucuronate (20α-PG, **28**) and 5β-pregnane-3α,17α,20β-triol-3α-glucuronate (20β–PG, **29**). Synthetic copies from the precursor 3α, 17-dihydroxy-5β-pregnan-20-one showed the same retention time on LC-MS and chemical shifts on NMR spectra, confirming the proposed structures. Bioassays confirmed the epimers were potent odourants and elicited an almost ten-fold increase in secretion of 17,20β-P by females, supporting the hypothesis that the epimers guide dominance hierarchies and spawning synchronization.[105] Notably, the discovery that the pheromone occurs as two epimers raises questions regarding the importance of chiral mixtures, which influence pheromone activity in insects.[110] LC-MS quantification of the epimers unveiled a ratio of approximately 20: 1 for 20β–PG: 20α–PG in dominant males, although a 4:1 mixture elicited a response in the bioassay. Enabled by bioassay-guided fractionation, characterization of the Mozambique tilapia male pheromone generates interest due to the novelty of the compounds and the opportunity for future studies on ratios of stereoisomers that may act as pheromones.[111]

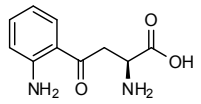
3.4 An amino acid guides mate search in masu salmon

Fig. 7 Masu salmon (*Oncorhynchus masou,* Wikipedia)

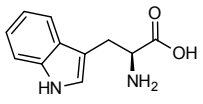
Female masu salmon (*Oncorhynchus masou*, Fig. 7) release a pheromone in their urine that attracts males.[112] Female mating pheromones in teleosts are arguably the best understood pheromones in fish, and a wealth of research indicates their active components are often sex steroids and prostaglandins.[32] Unexpectedly, bioassay-guided fractionation of urine from female masu salmon yielded the first non-hormonal mating pheromone in a teleost.[56]

Fractionation of urine guided by behavioural assays suggest the active component of the female pheromone is an amino acid.[56] A polystyrene resin column and step-wise ethanol elution separated female urine into fractions and bioassays tested the resulting

fractions for bioactivity. Gel filtration on a Sephadex LH-20 column further separated the active fraction and reverse-phase HPLC yielded a single purified compound. NMR, a Marfey's analysis, and comparisons with commercially available standards indicated the structure was L-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid (L-kynurenine, **30**). Lastly, laboratory bioassays confirmed the activity of L-kynurenine and inactivity of its precursor L-tryptophan and the epimer D-kynurenine (**31**). Our understanding of L-kynurenine, and other compounds, as a pheromone would benefit from follow-up behavioural tests, for example, in anadromous males. Although L-kynurenine was not novel and occurs, for example, as lens pigment from deep-sea fish *Stylephorus chordatus*,[113, 114] it was the first example of an amino acid pheromone and non-hormonal mating pheromone in a teleost. Consistent with other cases of bioassay-guided fractionation, characterization of the Masu salmon pheromone underscores the apparent lack of a consistent template for fish pheromones.



L-kynurenine (**30**)



L-tryptophan (**31**)

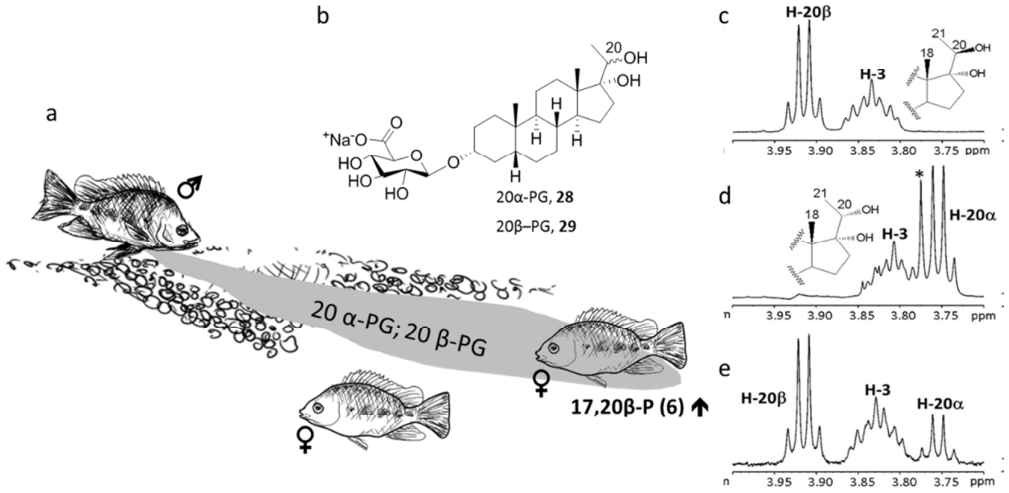


Fig. 6 Bioassay-guided fractionation of a male Mozambique Tilapia (*Oreochromis mossambicus*) pheromone. a) males release a large amount of urine with high concentrations of 20α- and 20β-pregnanetriol 3-glucuronates (20 α-PG and 20 β-PG) that are known to stimulate the maturation induction hormone, 17,20β-P, in females. b) Molecular structure of 5β-pregnane-3α,17α,20-triol 3α-glucuronate, no configuration shown on C-20. Extensions of representative ^{1}H NMR (500 MHz, MeOH-d_4) spectral regions (3.70-4.00 ppm) of c) synthetic sodium 20β-PG; d) synthetic sodium 20α-PG; e) separated fraction by HPLC, indicating both epimers present in the most active male urine fraction.

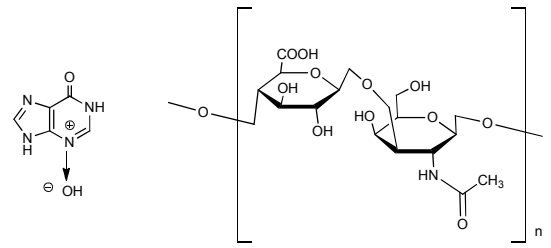
Fig. 8 Zebrafish (*Danio rerio*, Wikipedia)

3.5 Chondroitins elicit alarm responses in zebrafish

Upon injury, many fishes release an odour — termed *Schreckstoff* by Karl von Frisch[115] — that elicits an alarm response in conspecifics. Notably, the evolution of alarm pheromones remains unclear[116, 117] and they may or may not fit in our definition of pheromones. Regardless, the attempts to characterize alarm cues in fish mirror those focused on pheromones and provide another useful case study for our discussion of natural product chemistry and fish pheromone research. Research on the chemical identity of alarm pheromones indicates bioactivity of various small chemical structures, such as hypoxanthine-3-N-oxide (H3NO, **32**).[117, 118] Indeed, the majority of olfactory stimuli in fish are relatively small,[21] though peptides and proteins might also elicit olfactory and behavioural responses in fish.[119-121] Recently, various chemical analyses guided by bioassays on zebrafish (*Danio rerio*, Fig. 8) provided rare evidence for a macromolecular fish pheromone.[122]

Bioassay guided fractionation indicates glycosaminoglycan (GAG) chondroitin is a component of a mixture that elicits an alarm response in zebrafish.[122] Initial behavioural assays

indicated that H3NO only partially replicated the reaction of zebrafish to skin extract and initiated subsequent fractionation and other chemical analyses of skin extract in search of additional pheromone components. Pilot studies using hydrophilic columns revealed that the active components are highly polar. Anion-exchange chromatography followed by high-resolution gel-filtration yielded two fractions with high and low molecular weights, each of which elicited distinct alarm responses. Further analysis of the high molecular weight fraction eliminated proteins and lipids as candidates using pronase and peptidase treatments, and Folch's extractions. Likewise, phenol-sulphuric acid, alcian blue, and Elson-Morgan assays indicated GAGs occurred in the skin mucous and that their concentrations correlated with the behavioural responses. Finally, extended treatment of skin extract with chondroitinases eliminated its bioactivity, and commercial shark chondroitins (**33**) replicated some aspects of the bioactivity of raw skin extract. Although Mathuru et al. did not identify a specific compound and the structures of alarm pheromones remain elusive, their results offer evidence that macromolecules can act as fish pheromones. The chemistry-driven approach, although unique from those taken in the previous case studies, again implicated that pheromone structures is predictable.



H3NO (**32**) chondroitin (one unit in chain, **33**)

4. Concluding remarks

Bioassay-guided fractionation is a productive approach to identifying the pheromones used by fish. Although bioassay-guided fractionation is commonplace in insect research, many studies on fish pheromones test commercially available compounds suspected of pheromone function based on their biology. Such targeted screening pioneered the field, but the identification of novel and unexpected molecules as pheromones illustrates the difficulty in predicting the chemical structures of fish pheromones. Indeed, the pheromones that influence sexual behaviour show no obvious template; the bioactive compounds include sex steroids, prostaglandins, bile acids, amino acids, and a neurotoxin. With these compounds coming from only a few species and one ecological function, there is no telling the diversity of molecules that might emerge as pheromones using bioassay-driven fractionation.

We expect that technological advances will accelerate the pace at which new pheromones are identified. For example, mass spectrometry and NMR techniques have improved through advances in hardware, software, and pulse programs. NMR using only nanomoles of compound [123, 124] allow structure elucidation with the minute quantities often available in pheromone research. Sponge crystallography[125, 126] overcomes the requirement that a compound be crystallized prior to X-ray crystallographic analysis. In addition to advancing methods related to bioassay-guided fractionation, alternative methods including metabolomics,[61] genomics,[127] and others are likely to emerge as tools for identifying fish pheromones. Advances in techniques for identifying pheromones will subsequently accelerate research on pheromone biology.

Pheromones generate broad interest as a fruitful source of natural products, important signals for communication, and tools for managing animal populations.[1] Organisms use a staggering diversity of molecules to communicate,[3, 4, 128] of which we only highlight a few and only in fish. Pheromones are among the most important signals used across taxa,[1] and characterizing fish pheromones provides useful insight into the ecology and evolution of animal signals. Lastly, identified pheromones are promising tools with which to manage populations of fish that are invasive or in need of restoration.[129, 130]

5. Acknowledgements

The authors thank the Great Lakes Fishery Commission, Great Lakes Fishery Trust, National Science Foundation, and National Institute of Health for funding in their research of pheromones in fish. Anne Scott and two anonymous reviewers provided valuable suggestions on a draft of the manuscript.

6. References

1. T. D. Wyatt, *Pheromones and animal behavior: chemical signals and signatures*, Cambridge University Press, 2014.
2. P. W. Sorensen and B. D. Wisenden, *Fish Pheromones and Related Cues*, John Wiley & Sons, 2015.
3. S. F. Cummins and J. H. Bowie, *Nat. Prod. Rep.*, 2012, **29**, 642-658.
4. P. J. Apps, P. J. Weldon and M. Kramer, *Nat. Prod. Rep.*, 2015, **32**, 1131-1153.
5. P. Karlson and M. Lüscher, *Nature*, 1959, **183**, 55-56.
6. T. D. Wyatt, in *Chemical communication in crustaceans*, Springer, 2010, pp. 23-38.
7. C. D. Derby and P. W. Sorensen, *J. Chem. Ecol.*, 2008, **34**, 898-914.
8. T. J. Buchinger, M. J. Siefkes, B. S. Zielinski, C. O. Brant and W. Li, *Front. Zool.*, 2015, **12**, 32.
9. J. Teeter, *Can. J. Fish Aquat. Sci.*, 1980, **37**, 2123-2132.
10. L. A. Vrieze and P. W. Sorensen, *Can. J. Fish Aquat. Sci.* , 2001, **58**, 2374-2385.
11. C. M. Wagner, M. B. Twohey and J. M. Fine, *Anim. Behav.*, 2009, **78**, 593-599.

12. C. M. Wagner, E. M. Stroud and T. D. Meckley, *Can. J. Fish. Aquat. Sci.*, 2011, **68**, 1157-1160.
13. J. B. Hume, T. D. Meckley, N. S. Johnson, T. M. Luhring, M. J. Siefkes and C. M. Wagner, *Can. J. Fish. Aquat. Sci.*, 2015, **72**, 1799-1806.
14. R. T. Di Rocco, I. Imre, N. S. Johnson and G. E. Brown, *Hydrobiologia*, 2016, **767**, 279-287.
15. M. J. Siefkes, S. R. Winterstein and W. Li, *Anim. Behav.*, 2005, **70**, 1037-1045.
16. N. S. Johnson, A. Muhammad, H. Thompson, J. Choi and W. Li, *Behav. Ecol. Sociobiol.*, 2012, **66**, 1557-1567.
17. Y. W. Chung-Davidson, H. Y. Wang, M. J. Siefkes, M. B. Bryan, H. Wu, N. S. Johnson and W. M. Li, *BMC Neurosci.*, 2013, **14**.
18. K. Perrault, I. Imre and G. Brown, *Can. J. zool.*, 2014, **92**, 443-447.
19. C. Wagner, K. Kierczynski, J. Hume and T. Luhring, *J. Fish Biol*, 2016, **89**, 1897-1904.
20. R. Rodríguez‐Muñoz, A. Nicieza and F. Brana, *Funct. Ecol.*, 2003, **17**, 403-408.
21. T. J. Hara, *Rev. Fish Biol. Fish.*, 1994, **4**, 1-35.
22. K. B. Doving, R. Selset and G. Thommesen, *Acta Physiol. Scand.*, 1980, **108**, 123-131.
23. C. N. Polkinghorne, J. M. Olson, D. G. Gallaher and P. W. Sorensen, *Fish Physiol. Biochem.*, 2001, **24**, 15-30.
24. W. Li, A. P. Scott, M. J. Siefkes, H. Yan, Q. Liu, S.-S. Yun and D. A. Gage, *Science*, 2002, **296**, 138-141.
25. P. Sorensen, T. Hara, N. Stacey and F. W. Goetz, *Biol. Reprod*, 1988, **39**, 1039-1050.
26. K. Kawabata, *Amino Acids*, 1993, **5**, 323-327.
27. P. W. Sorensen, T. J. Hara, N. E. Stacey and J. G. Dulka, *J. Comp. Physiol. A*, 1990, **166**, 373-383.
28. E. Mollo, A. Fontana, V. Roussis, G. Polese, P. Amodeo and M. T. Ghiselin, *Frontiers in chemistry*, 2014, **2**, 92.
29. E. Mollo, M. Garson, G. Polese, P. Amodeo and M. Ghiselin, *Nat. Prod. Rep.*, 2017, **34**, 496-513.
30. L. Colombo, A. Marconato, P. C. Belvedere and C. Friso, *Ital. J. Zool.*, 1980, **47**, 355-364.
31. R. v. D. Hurk and J. Lambert, *Can. J. Zool.*, 1983, **61**, 2381-2387.
32. N. Stacey, *Fish Pheromones and Related Cues*, 2015, 33-88.
33. E. Christiansen and K. B. Døving, *Behav. Biol.*, 1976, **17**, 263-266.
34. J. Kittredge and F. Takahashi, *J. Theor. Biol.*, 1972, **35**, 467-471.
35. C. W. Appelt and P. W. Sorensen, *Anim. Behav.*, 2007, **74**, 1329-1338.
36. E. N. Barata, P. C. Hubbard, O. G. Almeida, A. Miranda and A. V. Canário, *BMC Biol.*, 2007, **5**, 54.
37. G. G. Rosenthal, J. N. Fitzsimmons, K. U. Woods, G. Gerlach and H. S. Fisher, *PLoS One*, 2011, **6**, e16994.
38. A. Scott and E. Vermeirssen, *Perspectives in comparative endocrinology*, 1994, 645-654.
39. U. S. v. Euler, *Klinische Wochenschrift*, 1935, **14**, 1182-1183.
40. M. W. Goldblatt, *J. Physiol.*, 1935, **84**, 208-218.
41. R. Eliasson, *Acta Physiol. Scand.*, 2016, **46**, 1-73.
42. Y. Yabuki, T. Koide, N. Miyasaka, N. Wakisaka, M. Masuda, M. Ohkura, J. Nakai, K. Tsuge, S. Tsuchiya and Y. Sugimoto, *Nat. Neurosci.*, 2016, **19**, 897.
43. L. R. Hagey, P. R. Moller, A. F. Hofmann and M. D. Krasowski, *Physiol. Biochem. Zool.*, 2010, **83**, 308-321.
44. A. F. Hofmann, L. R. Hagey and M. D. Krasowski, *J. Lipid Res.*, 2010, **51**, 226-246.
45. S. M. Houten, M. Watanabe and J. Auwerx, *EMBO J.*, 2006, **25**, 1419-1425.
46. A. Hofmann and L. Hagey, *Cell. Mol. Life Sci.*, 2008, **65**, 2461-2483.
47. T. J. Buchinger, W. Li and N. S. Johnson, *Chem. Senses*, 2014, **39**, 647-654.
48. K. B. Døving, R. Selset and G. Thommesen, *Acta Physiol.*, 1980, **108**, 123-131.
49. W. Li, P. W. Sorensen and D. D. Gallaher, *J. Gen. Physiol.*, 1995, **105**, 569-587.
50. R. Bjerselius, W. Li, J. H. Teeter, J. G. Seelye, P. B. Johnsen, P. J. Maniak, G. C. Grant, C. N. Polkinghorne and P. W. Sorensen, *Can. J. Fish Aquat. Sci.*, 2000, **57**, 557-569.
51. P. W. Sorensen, J. M. Fine, V. Dvornikovs, C. S. Jeffrey, F. Shao, J. Wang, L. A. Vrieze, K. R. Anderson and T. R. Hoye, *Nat. Chem. Biol.*, 2005, **1**, 324-328.
52. K. A. Jones, in *Fish chemoreception*, Springer, 1992, pp. 288-320.
53. J. Atema, *Fish Behaviour and its Use in the Capture and Culture of Fishes*, 1980, 57-101.
54. O. Kutsyna, Z. Velez, A. V. Canário, T. Keller-Costa and P. C. Hubbard, in *Chemical Signals in Vertebrates 13*, Springer, 2016, pp. 189-203.
55. T. Shoji, Y. Yamamoto, D. Nishikawa, K. Kurihara and H. Ueda, *Fish Physiol. Biochem.*, 2003, **28**, 249-251.
56. H. Yambe, S. Kitamura, M. Kamio, M. Yamada, S. Matsunaga, N. Fusetani and F. Yamazaki, *Proc. Natl. Acad. Sci. U.S.A.*, 2006, **103**, 15370-15374.
57. L. R. Hanson, P. W. Sorensen and Y. Cohenc, *Ann. N.Y. Acad. Sci.*, 1998, **855**, 521-524.
58. H. Zippel, A. Hansen and J. Caprio, *J. Comp. Physiol. A*, 1997, **181**, 425-437.
59. K. Matsumura, *Nature*, 1995, **378**, 563.
60. N. S. Johnson and W. M. Li, *J. Comp. Physiol.*, 2010, **196**, 701-711.
61. C. Kuhlisch and G. Pohnert, *Nat. Prod. Rep.*, 2015, **32**, 937-955.
62. S. Steiger, W. Haberer and J. K. Müller, *Biol. Lett.*, 2011, **7**, 822-824.
63. C. Sola and L. Tosi, *Environ. Biol. Fishes*, 1993, **37**, 197-204.
64. R. Madsen, T. Lundstedt and J. Trygg, *Anal. Chim. Acta*, 2010, **659**, 23-33.
65. J. G. Bundy, M. P. Davey and M. R. Viant, *Metabolomics*, 2009, **5**, 3.
66. A. Roux, D. Lison, C. Junot and J.-F. Heilier, *Clin. Biochem.*, 2011, **44**, 119-135.
67. L. M. Samuelsson and D. J. Larsson, *Mol. BioSyst.*, 2008, **4**, 974-979.
68. E. K. Prince and G. Pohnert, *Anal. Bioanal. Chem.*, 2010, **396**, 193-197.
69. R. X. Poulin, S. Lavoie, K. Siegel, D. A. Gaul, M. J. Weissburg and J. Kubanek, *Proceedings of the National Academy of Sciences*, 2018, DOI: 10.1073/pnas.1713901115.
70. J. M. Fine and P. W. Sorensen, *J. Chem. Ecol.*, 2008, **34**, 1259-1267.

71. M. Stewart and P. W. Sorensen, *Fish Pheromones and Related Cues*, 2015, 197-216.
72. M. Stewart and C. F. Baker, *J. Chem. Ecol.*, 2012, **38**, 135-144.
73. A. C. Burns, P. W. Sorensen and T. R. Hoye, *Steroids*, 2011, **76**, 291-300.
74. M. Kobayashi, P. W. Sorensen and N. E. Stacey, *Fish Physiol. Biochem.*, 2002, **26**, 71-84.
75. N. Stacey and P. W. Sorensen, in *Encyclopedia of Fish Physiology*, Academic Press, San Diego, 2011, pp. 1553-1562.
76. P. W. Sorensen, J. M. Fine, V. Dvornikovs, C. S. Jeffrey, F. Shao, J. Wang, L. A. Vrieze, K. R. Anderson and T. R. Hoye, *Nat. Chem. Biol.*, 2005, **1**, 324.
77. T. R. Hoye, V. Dvornikovs, J. M. Fine, K. R. Anderson, C. S. Jeffrey, D. C. Muddiman, F. Shao, P. W. Sorensen and J. Wang, *J. Org. Chem.*, 2007, **72**, 7544-7550.
78. S. L. Wehrli, K. S. Moore, H. Roder, S. Durell and M. Zasloff, *Steroids*, 1993, **58**, 370-378.
79. K. S. Moore, S. Wehrli, H. Roder, M. Rogers, J. N. Forrest, D. McCrimmon and M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**, 1354-1358.
80. T. D. Meckley, C. M. Wagner and M. A. Luehring, *J. Chem. Ecol.*, 2012, **38**, 1062-1069.
81. T. D. Meckley, C. M. Wagner and E. Gurarie, *Can. J. Fish Aquat. Sci.*, 2014, **71**, 533-544.
82. K. Li, C. O. Brant, M. Huertas, S. K. Hur and W. Li, *Org. Lett.*, 2013, **15**, 5924-5927.
83. K. Li, M. Huertas, C. Brant, Y.-W. Chung-Davidson, U. Bussy, T. R. Hoye and W. Li, *Org. Lett.*, 2015, **17**, 286-289.
84. K. Li, C. O. Brant, U. Bussy, H. Pinnamaneni, H. Patel, T. R. Hoye and W. M. Li, *Molecules*, 2015, **20**, 5215-5222.
85. J. A. Dale and H. S. Mosher, *J. Am. Chem. Soc.*, 1973, **95**, 512-519.
86. T. R. Hoye, C. S. Jeffrey and F. Shao, *Nat. Protoc.*, 2007, **2**, 2451-2458.
87. J. K. Rupprecht, Y. H. Hui and J. L. McLaughlin, *J. Nat. Prod.*, 1990, **53**, 237-278.
88. A. Boyer, *J. Org. Chem.*, 2015, **80**, 4771-4775.
89. S. Gahalawat, Y. Garg and S. K. Pandey, *Asian J. Org. Chem.*, 2015, **4**, 1025-1029.
90. U. Nookaraju and P. Kumar, *RSC Advances*, 2015, **5**, 63311-63317.
91. V. Mullapudi and C. V. Ramana, *Tetrahedron Lett.*, 2015, **56**, 3933-3935.
92. V. V. Reddy and B. V. S. Reddy, *Helv. Chim. Acta*, 2016, **99**, 636-641.
93. W. Li, 1994.
94. K. Li, C. O. Brant, M. J. Siefkes, H. G. Kruckman and W. Li, *PloS one*, 2013, **8**, e68157.
95. K. Li, A. M. Scott, C. O. Brant, S. D. Fissette, J. J. Riedy, T. R. Hoye and W. Li, *Org. Lett.*, 2017.
96. K. Li, A. M. Scott, J. J. Riedy, S. Fissette, Z. E. Middleton and W. Li, *J. Chem. Ecol.*, 2017, **43**, 543-549.
97. N. S. Johnson, S.-S. Yun and W. Li, *J. Chem. Ecol.*, 2014, **40**, 1152-1160.
98. M. Norlin and K. Wikvall, *Curr. Mol. Med.*, 2007, **7**, 199-218.
99. D. W. Russell, *Annu. Rev. Biochem*, 2003, **72**, 137-174.
100. W. H. Elliott and P. M. Hyde, *The American journal of medicine*, 1971, **51**, 568-579.
101. J. K. Reddy and T. Hashimoto, *Annu. Rev. Nutr.*, 2001, **21**, 193-230.
102. C. O. Brant, Y. W. Chung-Davidson, K. Li, A. M. Scott and W. M. Li, *BMC Biochem.*, 2013, **14**.
103. K. Li, C. O. Brant, M. J. Siefkes, H. G. Kruckman and W. M. Li, *Plos One*, 2013, **8**, e68157.
104. G. Haslewood and L. Tökés, *Biochem. J*, 1969, **114**, 179-184.
105. T. Keller-Costa, P. C. Hubbard, C. Paetz, Y. Nakamura, J. P. da Silva, A. Rato, E. N. Barata, B. Schneider and A. V. Canario, *Curr. Biol.*, 2014, **24**, 2130-2135.
106. M. Huertas, O. G. Almeida, A. V. M. Canário and P. C. Hubbard, *Gen. Comp. Endocrinol.*, 2014, **196**, 106-111.
107. T. Keller-Costa, A. V. Canário and P. C. Hubbard, *J. Exp. Biol.*, 2014, **217**, 4203-4212.
108. P. Frade, P. Hubbard, E. Barata and A. V. Canario, *J. Fish Biol.*, 2002, **61**, 1239-1254.
109. E. N. Barata, J. M. Fine, P. C. Hubbard, O. G. Almeida, P. Frade, P. W. Sorensen and A. V. Canário, *J. Chem. Ecol.*, 2008, **34**, 438-449.
110. K. Mori, *Biorg. Med. Chem.*, 2007, **15**, 7505-7523.
111. W. Li and T. Buchinger, *Curr. Biol.*, 2014, **24**, R843-R845.
112. H. Yambe and F. Yamazaki, *J. Fish Biol.*, 2000, **57**, 1058-1064.
113. A. Thorpe, R. J. W. Truscott and R. H. Douglas, *Exp. Eye Res.*, 1992, **55**, 53-57.
114. A. Chiarugi, E. Rapizzi, F. Moroni and F. Moroni, *FEBS Lett.*, 1999, **453**, 197-200.
115. K. von von Frisch, *Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung*, Springer-Verlag, 1941.
116. R. J. F. Smith, *Rev. Fish Biol. Fish.*, 1992, **2**, 33-63.
117. M. C. Ferrari, B. D. Wisenden and D. P. Chivers, *Can. J. Zool.*, 2010, **88**, 698-724.
118. K. B. Døving and S. Lastein, *Ann. N.Y. Acad. Sci.*, 2009, **1170**, 413-423.
119. Ô. Andersen and K. B. Deving, *Neuroreport*, 1991, **2**, 458-460.
120. B. D. Wisenden, M. L. Rugg, N. L. Korpi and L. C. Fuselier, *Behaviour*, 2009, **146**, 1423-1442.
121. C. Hinz, *Sci. Rep.*, 2013, **3**.
122. A. S. Mathuru, C. Kibat, W. F. Cheong, G. Shui, M. R. Wenk, R. W. Friedrich and S. Jesuthasan, *Curr. Biol.*, 2012, **22**, 538-544.
123. T. F. Molinski, *Curr. Opin. Biotechnol.*, 2010, **21**, 819-826.
124. T. F. Molinski, *Nat. Prod. Rep.*, 2010, **27**, 321-329.
125. Y. Inokuma, T. Arai and M. Fujita, *Nat. Chem.*, 2010, **2**, 780-783.
126. S. Yoshioka, Y. Inokuma, M. Hoshino, T. Sato and M. Fujita, *Chem. Sci.*, 2015, **6**, 3765-3768.
127. J. R. Doroghazi, J. C. Albright, A. W. Goering, K.-S. Ju, R. R. Haines, K. A. Tchalukov, D. P. Labeda, N. L. Kelleher and W. W. Metcalf, *Nat. Chem. Biol.*, 2014, **10**, 963-968.
128. M. R. Symonds and M. A. Elgar, *Trends Ecol. Evol.*, 2008, **23**, 220-228.
129. P. W. Sorensen, *Fish Pheromones and Related Cues*, 2015, 255-268.
130. H. A. Dawson, M. L. Jones, B. J. Irwin, N. S. Johnson, M. C. Wagner and M. D. Szymanski, *Nat. Resour. Model.*, 2016.