according to their past experience. A decision rule specifies the trait (laying date, in this case) as a function of an estimate of future environmental suitability made at the time of making the decision. Such a rule may be genetically determined, but birds might be expected to recalibrate this rule if it appears to produce a mismatch between breeding and the conditions for feeding the young (*17*, *18*) (in this case, a temporal mismatch between nestling phase and caterpillar peak date).

Learning when is best to breed is only adaptive when the environment in a certain locality carries some information on the environment at the time of the next breeding event. The shifts in laying date shown in this study may be viewed as a mechanism by which birds adapt their breeding time to the local environmental conditions. This is particularly relevant for species such as the blue tit that may settle in a wide variety of habitats, but once settled will breed at that same location for the duration of their lives. The seven localities on the Hoge Veluwe, for which we measured caterpillar biomass patterns for 1993 to 2000, differed in the peak date of caterpillar biomass (effect of locality on variation in local peak dates across 8 years: $F_{7,42} = 11.0$, $P < 0.0001$ corrected for year). This indicates that some sites are consistently earlier than others, independent of the between-year differences. If the best time for rearing the offspring (i.e., the caterpillar peak date) in a certain locality is consistently earlier or later than in other localities, birds are expected to benefit from learning.

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8. Mealworms (*Tenebrio molitor*) and larvae of the waxmoth (*Galleria mellonella*) were placed in small trays inside the nest box from the day of hatching of the first egg to the day of fledging of the young in the breeding seasons 1997 to 1999. Feeding trays were placed near the entrance hole, so that only the adults could take the food. In the first 6 days after hatching, food consisted of a mixture of the two species, whereas afterward it was composed of mealworms only. The amount offered increased approximately linearly from 1.0 g/day on day 0 to 20 g/day on day 10 after hatching and then leveled off at 20 g/day for a 12-chick brood. Proportional adjustments in quantity were made for smaller and larger broods. Remaining food was replaced each day with the scheduled amount. Half of the nests were food-supplemented, whereas the other half served as a control. Treatments were chosen within pairs of nests with similar hatching dates. An effort was made to assign different treatment levels to nests in similar habitats.
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16. For each female that laid in 2 successive years we calculated (i) the difference in laying date between the 2 years (laying $\text{date}_{\text{year 2}}$ – laying $\text{date}_{\text{year 1}}$) and (ii) the between-year difference in the mean laying date of the females other than the 15 experimental ones (Fig. 1) laying in nest boxes, within a radius of 200 m from the focal nest box of the female (mean laying $\text{date}_{\text{year 2}}$ – mean laying $\text{date}_{\text{year 1}}$). This difference is an estimate of the change in the environment experienced by the focal females (*6*). On average, we used 2.8 ± 1.0 (SD) neighbor nests for each focal nest. Control and food-supplemented nests did not differ in the average laying date of neighbor nests (year 1, $F_{1,11} = 2.13$ and $P = 0.17$; year 2, $F_{1,11} = 1.64$ and $P = 0.23$, controlling for year). We calculated the regression of between-year difference in laying date on the difference in mean laying date of neighbors ($R^2 = 0.51$, $n = 15$; $F_{1,13} = 13.59$, $P = 0.003$), so that its residuals were the between-year changes in laying date of individual females independent of the neighbors. The degree of synchronization between tits' breeding and the caterpillars was defined as the difference in days between day 10 of the tits' nestling period (approximately the midpoint of the nestling period) and the caterpillar peak date. For this latter, we used the average of the study area because the seven sampling sites did not coincide with blue tit territories. Throughout the text, food-supplemented females refer to females receiving food in the nestling phase.
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Bile Acid Secreted by Male Sea Lamprey That Acts as a Sex Pheromone

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We show that reproductively mature male sea lampreys release a bile acid that acts as a potent sex pheromone, inducing preference and searching behavior in ovulated female lampreys. The secreted bile acid 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate was released in much higher amounts relative to known vertebrate steroid pheromones and may be secreted through the gills. Hence, the male of this fish species signals both its reproductive status and location to females by secreting a pheromone that can act over long distances.

The sea lamprey, *Petromyzon marinus*, is an ancestral jawless fish and an invasive parasite of fishes, particularly in the Laurentian Great Lakes of North America. It migrates into streams to spawn in the spring. The males arrive earlier than the females (*1*) and build nests in areas where flow rates are 0.5 to 1.5 m s^{-1} (*1*, *2*). It has long been suspected that the males release a pheromone to guide the females to their nests (*3*, *4*). This type of sex pheromone, capable of inducing spatial orientation of conspecifics "downwind," is well established in insects (*5*), but not so in vertebrates, whose identified sex pheromones tend to have a small

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range of effectiveness (*6–12*). In fish, the known sex pheromones are gonadal steroids or prostaglandins and have been identified from a priori knowledge of their structures (*9–12*). However, it has not been clear whether such sex pheromones can function at great distances for fish such as the sea lamprey.

Behavioral tests confirmed that water conditioned by spermiating lamprey males influenced the distribution and locomotor activities of ovulated female lampreys. When tested in a two-choice maze (*13*), ovulated females (but not males or preovulatory females) (*14*) spent more time in the compartment conditioned with washings from spermiating males (Table 1). There was no preference of ovulated females for washings of prespermiating males or females (*14*). Further, the ovulated females showed increased search behavior in the chamber with water conditioned by spermiating males (Table 1). At a natural spawning site, ovulated females that were tagged with radio transmitters (*15*) and placed 65 m downstream (*16*) showed a similar response ($P = 0.02$) (*14*), indicating a large active space for the male pheromone.

Water conditioned by spermiating lampreys was passed through C-18 solid phase extraction (SPE) cartridges (*17*), which can extract nearly 100% of attractant molecules, as determined by electro-olfactogram (EOG) experiments (*18*). In our maze (*13*), ovulated females spent more time and showed increased search behavior on the side conditioned with extracts from spermiating males (Table 1).

SPE extracts were subjected to fast atom bombardment mass spectrometry (FABMS) and thin-layer chromatography (TLC) to detect the compounds released by spermiating males and then were subjected to reverse-phase high-performance liquid chromatography (HPLC) to isolate them. FABMS identified an abundant ion representing the protonated molecule (MH^+) at a mass-to-charge ratio (*m/z*) of 473 in extracts from spermiating males. In the negative mode, a corresponding strong deprotonated molecule $[M-H]^-$ ion at *m/z* 471 was observed, suggesting the presence of an acidic moiety in the molecule (Fig. 1). Tandem MS analysis of this peak showed that it lost 98 mass units, suggesting that the compound was phosphorylated or sulfated. Similar ions were not present in detectable amounts in extracts from prespermiating males (Fig. 1, inset) or from females (*14*). TLC of extracts (*19*) displayed a relatively large amount of a few major compounds in spermiating, as opposed to prespermiating, male washings (Fig. 2, inset). The material separated into three bands on TLC, the one at the origin being established, by dilution, as the most abundant. The HPLC (*20*) fractions eluting at 46 and 47 min contained the 472-dalton molecule according to FABMS analysis, were stained strongly by phosphomolybdic acid (PBA) when spotted on TLC plates, remained at the origin when run on TLC, and had the highest olfactory potency according to an EOG (Fig. 2) (*21*). HPLC fractions of extracts of prespermiating male washings at 46 and 47 min did not contain the 472-dalton molecule and did not show EOG potency (*14*). Fractions 64 and 71 are unidentified.

The chemical structure of the 472-dalton molecule was determined by nuclear magnetic resonance spectrometry (*22*). The one-dimensional (1D) ^{13}C spectrum (Fig. 3A) showed one peak at 210.9 parts per million (ppm) and no other peaks above 80 ppm, suggesting the presence of a carbonyl group and the absence of double bonds between carbon atoms. The ^1H-^{13}C heteronuclear single-quantum coherence (HSQC) (Fig. 3B) showed three intense cross peaks characteristic of CH3 groups. Two of them were singlet peaks, suggesting they were bonded to quarternary carbons. CH and CH2 groups were distinguished by ^{13}C editing. The cross peaks with ^1H chemical shifts >3.0 ppm and ^{13}C chemical shifts >60 ppm were assigned to CH2 or CH groups linked to an oxygen through a single bond. These chemical groups were then linked together with through-bond correlations obtained from 2D ^1H-^1H correlated spectroscopy (COSY) and total correlation spectroscopy (TOCSY) and ^1H-^{13}C HSQC-TOCSY and heteronuclear multiple-bond correlation (HMBC) spectra. The stereochemistries of 7-H and 12-H were determined on the basis of their narrow multiplets (<10 Hz), and that of 5-H was determined on the basis of the chemical shift of C-19 (9.7 ppm) (*22*, *23*). The formula based on this structure, C24H40O7S, was confirmed by an exact mass measurement (MH^+ calculated, 473.2573; observed, 473.2578; error 1.1 ppm), indicating that the compound contained a sulfate rather than a phosphate group. We concluded that the structure was 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate.

The deduced structure differs from that of petromyzonol sulfate (3α,7α,12α,24-tetrahydroxy-5α-cholan 24-sulfate; PS) by its 3-keto, as opposed to 3α-hydroxyl, group. PS is a lamprey larvae bile acid (*21*, *24*) and a component of a pheromone that influences behaviors of migrating, but not reproductively mature, adult lampreys (*21*, *25*). We converted the 3α-OH of synthetic PS into 3-keto (*26*) and acquired its ^1H-^{13}C HSQC (*22*). The chemical shifts and intensity of cross peaks were virtually identical between the converted compound and purified male pheromone (Fig. 3B), suggesting that both molecules had an identical chemical structure and purity.

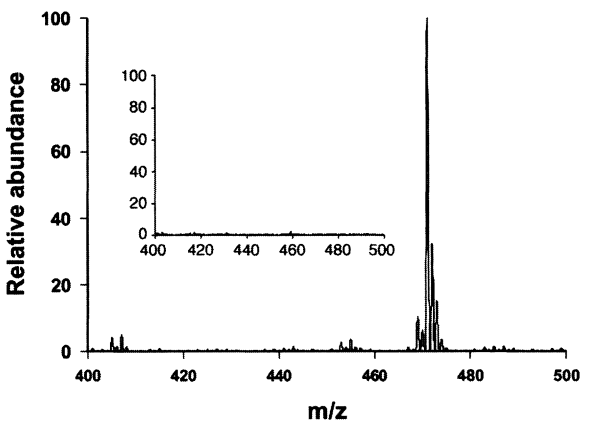


Fig. 1. Fast atom bombardment mass spectra of an extract of washings from a spermiating male sea lamprey and of an extract of washings from a prespermiating male (inset). The matrix is glycerol.

Table 1. Influence of male odorants on distribution and search behavior of ovulated female sea lampreys in a two-choice maze. "Conditioned" refers to the side of maze into which caged fish or test substances were placed; "unconditioned" refers to the control side. The numbers refer to the number of ovulated females that were tested and that spent more of their time (attraction) or showed more activity (search behavior) in either the conditioned or unconditioned sides (*13*). SM, spermiating male; PSM, prespermiating male; SMW, washings collected from spermiating males; SME, C-18 SPE extracts of spermating male washings; PP, purified pheromone (7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate). *P* values were determined with a Wilcoxon Signed Ranks Test (two-tailed) using indices of preference described in (*13*). *NS, not significant.

Stimuli	Attraction			Search behavior		
	Unconditioned	Conditioned	*P* value	Unconditioned	Conditioned	*P* value
SM	0	22	0.01	0	8	0.01
PSM	12	12	NS*	3	3	NS*
SMW	3	12	0.01	0	7	0.01
SME	3	11	0.01	2	7	0.05
PP	0	8	0.01	0	6	0.03

Further, these two compounds comigrated on TLC, co-eluted on HPLC, and showed tandem FABMS.

We confirmed that the purified compound, which showed a virtually identical ^{1}H-^{13}C HSQC to the synthetic compound, (Fig. 3B) replicated the pheromonal activity of washings of spermiating males. Approximately 30 mg of pheromone was isolated from 4-hour washings of approximately 30 spermiating males, suggesting a rate of release of about 250 μg male^{-1} hour^{-1}. From this, we estimated that, in the experiments with live males (*13*), the pheromone reached a concentration of between 0.1 and 0.2 nM. We therefore tested it in our maze (*13*) at a final concentration of 0.17 nM. Ovulated females spent a longer time and showed increased search behavior on the side containing the pheromone (Table 1).

To determine the site of release of the pheromone, we tested washings from bisected (*27*) male lampreys. Water conditioned by the head region induced a large EOG response at 10,000 times dilution, whereas the water conditioned by the posterior region did not induce a detectable response (*14*). Further, only water conditioned by the head region was attractive to ovulated females ($P < 0.01$) and, by SPE extraction and FABMS, was determined to contain the [M-H]$^-$ ion at a *m/z* of 471 (*14*).

The bile acid 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate was present in the liver of spermiating males (*14*), suggesting that that is where it is synthesized. It is unlikely that this bile acid is needed for lipid digestion, because adult lampreys do not feed, nor do they have bile ducts or gall bladders (*28*). Its delivery to the gills should be through the bloodstream. Because the hepatic veins carry blood directly to the heart, and because all the blood from the heart goes through the gills, its excretion is potentially very efficient. However, in elasmobranchs and teleosts (*27*, *29*), the passive transfer of sulfated compounds across gills is negligible. Lampreys likely overcome this problem using the profuse glandular cells present in the gills of spermiating males (*30*). Females do not develop these cells at any stage. It seems probable that these cells are responsible for the active excretion of the identified pheromone. If so, this suggests that male lampreys are "active signalers" rather than the females being "chemical spies," which is the current leading hypothesis concerning the evolution of fish sex pheromones (*31*).

The selection pressure favoring the evolution of a bile acid derivative, rather than a normal steroid or prostaglandin, as a sex attractant may have been the necessity to cover a large active space. Bile acids, in particular sulfated ones, are more water soluble and can be produced in larger quantities than steroids. A spermiating male lamprey (~250 g) releases sufficient amounts of this pheromone in 4 hours to be detectable by females when diluted in 10^7 liters of water (*18*, *32*). This volume is about 10^5 times greater than that (130 liters) of the

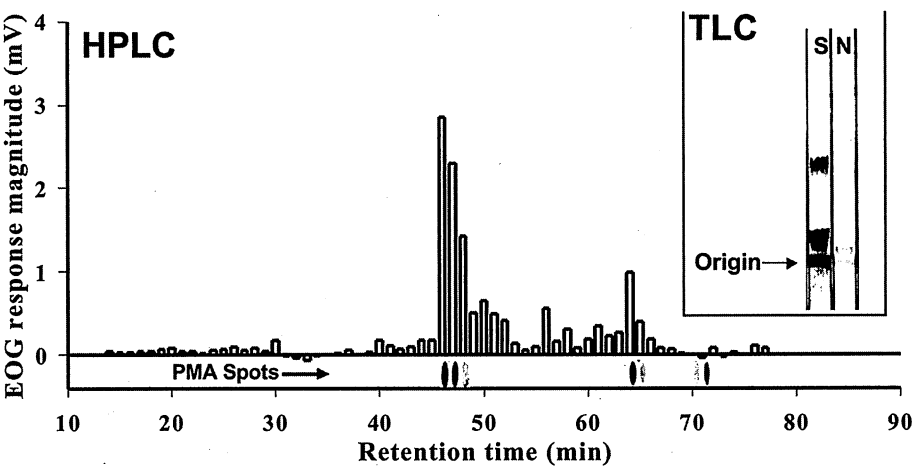
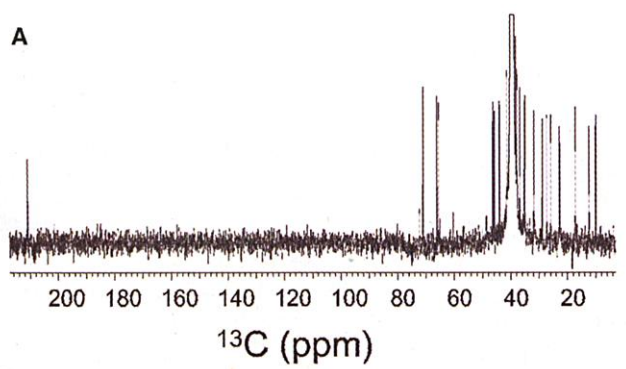


Fig. 2. Electro-olfactograghic potency of fractions derived from reverse-phase HPLC separation (*20*) of water extracted from five spermiating male lampreys. The response magnitude is in millivolts. The spots produced by staining of 5 μl of each fraction with phosphomolybdic acid (PMA) (*19*) are shown just below the ordinate. The inset shows TLC of equal amounts of extract from spermiating (S) and prespermiating (N) male lampreys after staining with PMA. The three TLC bands had the following correspondence to the HPLC fractions: origin = 46/47; slow-moving band = 71; fast-moving band = 64. The staining method is not quantitative; thus, the relative size of the TLC bands is not a true reflection of their relative abundance.



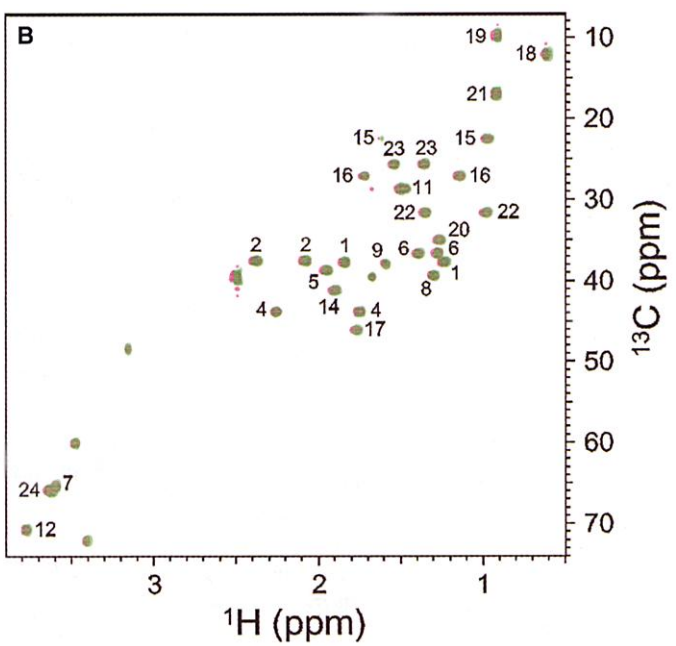


Fig. 3. 1D ^{13}C spectrum of the isolated male sea lamprey sex pheromone (**A**) and overlay of 2D ^{1}H-^{13}C HSQC spectra (**B**) of the isolated (red) and synthetic (green) male sea lamprey sex pheromone (*22*). The unlabeled cross peaks in the ^{1}H-^{13}C HSQC spectrum (B) are due to impurities or the solvent.

main gonadal (steroid) pheromone released by a 25-g female goldfish (*33*). Interference with this pheromone system offers an attractive target for selective and environmentally benign control of the sea lamprey, whose invasion of the Great Lakes represents arguably the worst ecological disaster ever to befall a large watershed (*34*).

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17. A lamprey was placed in 10 liters of aerated water for 4 hours and then removed. The water was drawn through a filter paper (Whatman No. 3) and then SPE cartridges (Sep-Pak; Waters Chromatography, Millipore, Milford, MA; prewashed with 5 ml of methanol, followed by 5 ml of distilled water) at a rate of up to 20 ml min^{-1}. One liter was pumped through each cartridge, which was then washed with 5 ml of distilled water and eluted with 5 ml of methanol.
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Functional Annotation of a Full-Length *Arabidopsis* cDNA Collection

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Full-length complementary DNAs (cDNAs) are essential for the correct annotation of genomic sequences and for the functional analysis of genes and their products. We isolated 155,144 RIKEN *Arabidopsis* full-length (RAFL) cDNA clones. The 3′-end expressed sequence tags (ESTs) of 155,144 RAFL cDNAs were clustered into 14,668 nonredundant cDNA groups, about 60% of predicted genes. We also obtained 5′ ESTs from 14,034 nonredundant cDNA groups and constructed a promoter database. The sequence database of the RAFL cDNAs is useful for promoter analysis and correct annotation of predicted transcription units and gene products. Furthermore, the full-length cDNAs are useful resources for analyses of the expression profiles, functions, and structures of plant proteins.

Arabidopsis thaliana has been adopted as a model organism in the study of plant biology because of its small size, short generation time, and high efficiency of transformation (*1*). To sequence its small genome [125 megabases (Mb)] (*2*), scientists in Japan, Europe, and the United States collaborated in the *Arabidopsis* genome sequencing project (*3*). Two of five chromosomes (chromosomes 2 and 4, except for the nucleolar organizer regions and centromeres) were sequenced in 1999 (*4*, *5*), and the remaining three