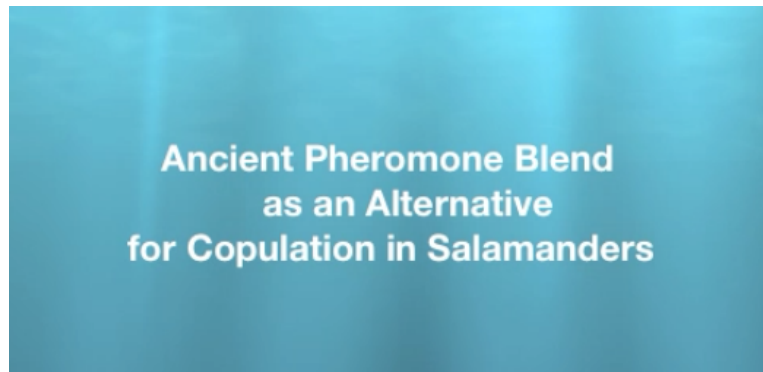


Electronic Supplementary Material

Movie S1. - Natural and experimental behaviour in palmate newts.



Movie can be seen at

<http://www.amphibia.be/downloads/abcde/pheromone.mov>

Figure S1. - SPF expression in courtship water during and at the end of the breeding season.

We used ion exchange chromatography followed by RP-HPLC to purify SPF proteins from courtship water and compared their diversity in courtship water taken during the highlight of the breeding season with that sampled towards the end of the breeding season (when males are still tail-fanning). Comparison of RP-HPLC elution profiles indicated that the SPF content is strongly reduced at the end of the breeding season (grey bar). Given that we compare molecules from courtship water, i.e. molecules that were effectively tail-fanned by the male to the female, this observation strengthens the evidence for a courtship function of SPF proteins during the breeding season.

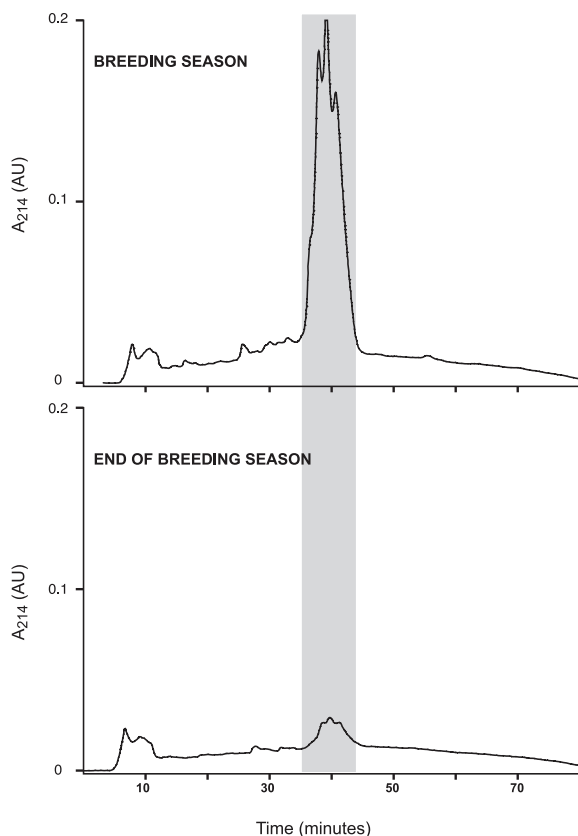


Figure S2. - In source fragmentation pattern of SPF used to determine post-translational modifications. Eight hexoses (Mr of one hexose: 162.14) and one core GlcNAc (Mr: 203.19) could be fragmented from a ten-times charged ion with 10 charges (m/z 2104.4). Taking into account the eight hexoses and two GlcNAcs, the m/z value of 2104.4 corresponds to the ion with 10 charges of SPF 1 ($[(2104.4 \times 10) - 10] - [8 \times 162.14] - [2 \times 203.19] = 19330.5$). Typically, N-linked glycans contain two HexNAcs. However, the glycosidic bond between the second core HexNAc and the protein moiety rarely breaks during in-source in fragmentation, thus no signal corresponding to the removal of the second core HexNAc was detected. The N-linked glycan structure is most likely of the high mannose type, more specifically a Man(9 or 8)GlcNAc(2)-glycan.

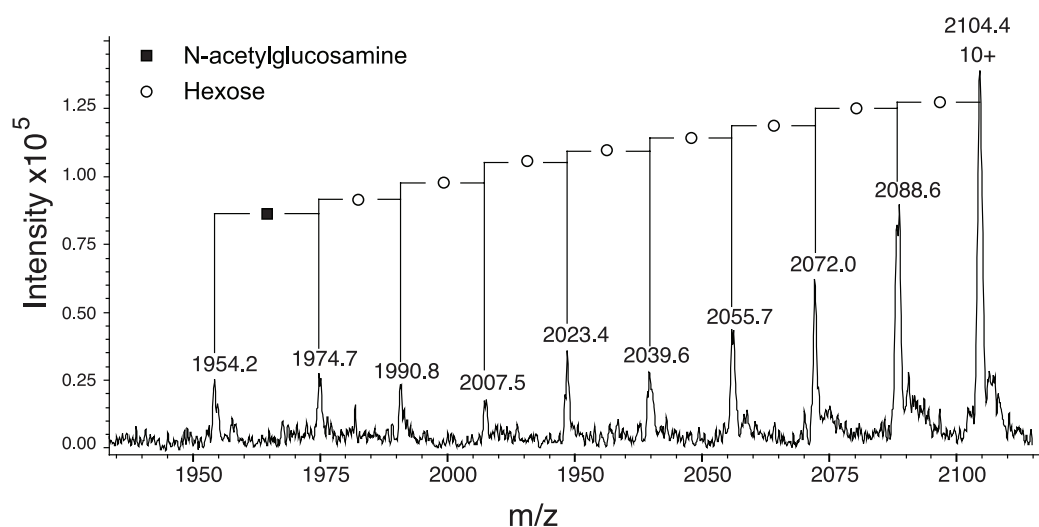


Table S1 - Results of behavioural experiments: All behavioural experiments are with *Lissotriton helveticus*. Stimuli are as follows: Lh = *Lissotriton helveticus* courtship water, Lv = *Lissotriton vulgaris* courtship water, Ia = *Ichthyosaura alpestris* courtship water; CP = *Lissotriton helveticus* courtship-specific peak, MF = non-courting male-female water; SPF-A = SPF 1 + SPF 3 glycoforms; SPF-B = SPF 3 glycoforms only. N = the number of couples tested. **Following:** f indicates the number of couples in which following behaviour was observed for at least 30 seconds. **Pointing:** Statistic analyses (stimulus 1 *versus* stimulus 2) compare the cumulative duration of pointing (s) per couple during 10 minutes of observation.

				Following		Pointing			
Species-specificity (see Figure 1b)									
stimulus 1	N1	stimulus 2	N2	f1	f2	Kruskal-Wallis	Mann-Whitney U	U	Z
Lh1	12	Control 1	11	9	1	<0.001	<0.01	23.000	-2.647
Lh1	12	Lv	12	9	4		0.065	40.000	-1.848
Lh1	12	Ia	12	9	0		<0.01	15.000	-3.291
Courtship peak (see Figure 1b)									
stimulus 1	N1	stimulus 2	N2	f1	f2	Kruskal-Wallis	Mann-Whitney U	U	Z
CP	10	Control 2	12	9	1	<0.001	<0.05	25.000	-2.308
CP	10	Lh2	13	9	11		0.385	51.000	-0.868
MF	11	Control 2	12	2	1		0.166	43.500	-1.386
Purified SPF (see Figure 2 d and e)									
stimulus 1	N1	stimulus 2	N2	f1	f2	Kruskal-Wallis	Mann-Whitney U	U	Z
SPF-A	10	Control 3	11	8	0	<0.01	<0.01	14.000	-2.888
SPF-B	10	Control 3	11	5	0		<0.05	24.500	-2.150

Table S2.- Edman sequences. Cysteines were not detected (indicated as X in the sequence) as they were not alkylated prior to the sequence analysis.

SPF in courtship peak (cf. Fig. 2C)	
SPF1	LLXEKXLVSGTTQXSGIFKQX
SPF2	LLXETXLASGTSQXS
SPF2	LLXETXLA
SPF3	IEXEVXSNRASLDXSGDLV
SPF5	ILXEKXLATSTTQXSXIFKQ
SPF5-like	ILSEK (T) FATSTTQ
SPF20	LLxEKxLASGTTQXS
SPF20	LLxEKxLASGTTQXSGIFKQ
Purified SPF (cf. Fig. 2D)	
SPF1	LLXEKXLVSGTTQXSGIFXQ
SPF3	IEXEVXSNRASL
SPF3	IEXEVXX
Purified SPF (cf. Fig. 2E)	
SPF3	IEXEVXSNRASLD

Table S3. - Protein masses in the courtship peak. Full analysis of MS-Data of SPF proteins in the courtship specific peak (fraction numbers refer to Figure 2c), with corresponding precursor sequence matches. The table states (i) the measured relative molecular masses (Mr), (ii) the calculated precursor masses taking into account disulfide bridges and removal of the signal peptide, and (iii) the calculated precursor masses with glycosylation, taking into account S-S bridges and removal of the signal peptide. None of the precursor amino acid (AA) sequences shows homology with the 10 AA fragment that codes for the attractant deca-peptide sodefrin in *Cynops pyrrhogaster*, indicating that palmate newts do not express such a peptide.

SPF found in courtship water (CP)		Match with precursor found in abdominal gland				
Fraction	i. Measured Mr	Precursor	ii. Calculated Mr precursor	GlcNAc (Mr 203.19)	Hexose (Mr 162.1442)	iii. Calculated Mr glycosylated precursor
47	20681.8	No match A				
47	20843.7	No match A + hex				
48	20830.0	SPF 2	19448.7	2	6	20828.0
48	20667.3	SPF 2	19448.7	2	5	20665.8
49	20830.4	SPF 2	19448.7	2	6	20828.0
49	20667.5	SPF 2	19448.7	2	5	20665.8
50	20829.7	SPF 2	19448.7	2	6	20828.0
50	20667.6	SPF 2	19448.7	2	5	20665.8
51	20697.3	No match B				
51	20828.4	SPF 2	19448.7	2	6	20828.0
51	20738.7	SPF 4	19358.6	2	6	20737.9
51	20667.0	SPF 2	19448.7	2	5	20665.8
52	20696.8	No match B				
52	20737.4	SPF 4	19358.6	2	6	20737.9
52	20896.5	SPF 5	19515.8	2	6	20895.0
52	21059.7	SPF 5	19515.8	2	7	21057.2
52	21028.2	No match C				
53	20897.6	SPF 5	19515.8	2	6	20895.0
53	21059.0	SPF 5	19515.8	2	7	21057.2
53	20865.8	No match C- hex				
53	20834.7	No match D				
53	21028.2	No match C				
53	20797.5	No match E				
53	20698.8	No match B				
54	20985.7	No match F				
54	20896.1	SPF 5	19515.8	2	6	20895.0
54	20868.2	No match C- hex				
54	20834.9	No match D				
55	21037.7	SPF 1	19331.8	2	8	21035.3
55	20981.2	No match F				
55	20874.2	SPF 1	19331.8	2	7	20873.2
55	20713.6	SPF 1	19331.8	2	6	20711.1
56	21037.6	SPF 1	19331.8	2	8	21035.4
56	20875.5	SPF 1	19331.8	2	7	20873.2
56	20713.1	SPF 1	19331.8	2	6	20711.1
57	20876.3	SPF 1	19331.8	2	7	20873.2
57	20924.7	No match G				
57	21038.4	SPF 1	19331.8	2	8	21035.4
57	20712.8	SPF 1	19331.8	2	6	20711.1
58	20876.1	SPF 1	19331.8	2	7	20873.2
58	20924.2	No match G				
58	21035.2	SPF 1	19331.8	2	8	21035.4
58	20712.3	SPF 1	19331.8	2	6	20711.1
59	20327.1	SPF 3	18945.2	2	6	20324.4
59	20164.5	SPF 3	18945.2	2	5	20162.3
60	20326.5	SPF 3	18945.2	2	6	20324.4
60	20163.7	SPF 3	18945.2	2	5	20162.3
60	20003.4	SPF 3	18945.2	2	4	20000.1
60	19679.1	SPF 3	18945.2	2	2	19675.8

Table S4. - Dating estimates and 95% highest posterior density (HPD). Node numbers correspond to the time tree of SPF protein diversification in Figure 3.

	Node	Mean	95% HPD
Duplication 1	1	288.4	200.6 - 385.1
Duplication 2	2	220.8	165.2 - 282.4
Plethodontidae vs. (Ambystomatidae, Salamandridae)	3	173.0	145.0 - 204.0
Ambystomatidae vs. Salamandridae	4	143.4	98.4 - 186.8
Duplication 3	5	121.9	71.7 - 175.5
Duplication 4	6	85.9	54.0 - 122.8
Duplication 5	7	73.3	45.3 - 103.1
Crown group Plethodontidae	8	70.6	41.7 - 101.2
<i>L. vulgaris</i> vs <i>L. montandoni</i>	9	16.3	7.2 - 27.0
<i>L. vulgaris</i> vs <i>L. montandoni</i>	10	13.2	6.1 - 21.2