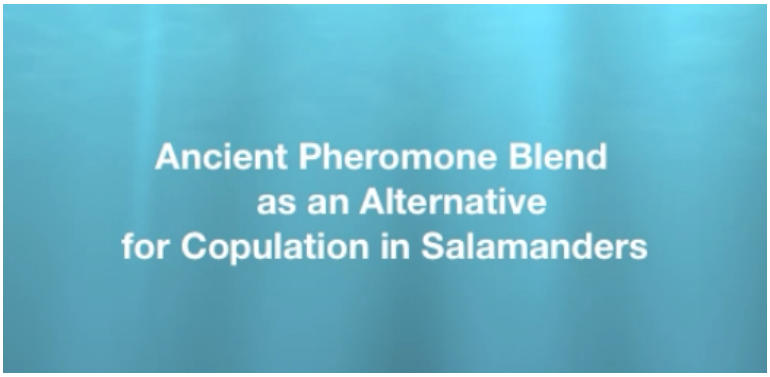
## Electronic Supplementary Material

**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 during and at the end of the breeding season**. We used ion exchange chromatography followed by RP-HPLC to purify SPF proteins 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. 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.

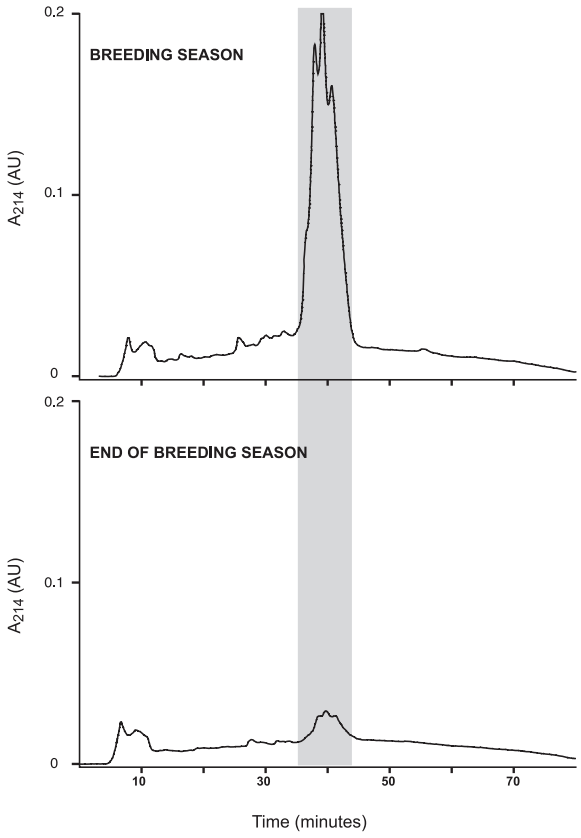


**Figure S2. - In source fragmentation pattern**. Eight hexoses (Mr of one hexose: 162.14) and one core GlcNAc (Mr: 203.19) could be fragmented from a ten-times charged ion with 10 charges (m/z 2104.4). Taking into account the eight hexoses and two GlcNAcs, the m/z value of 2104.4 corresponds to the ion with 10 charges of SPF 1 ( [[2104.4*10]-10]-[8*162.14]-[2*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.

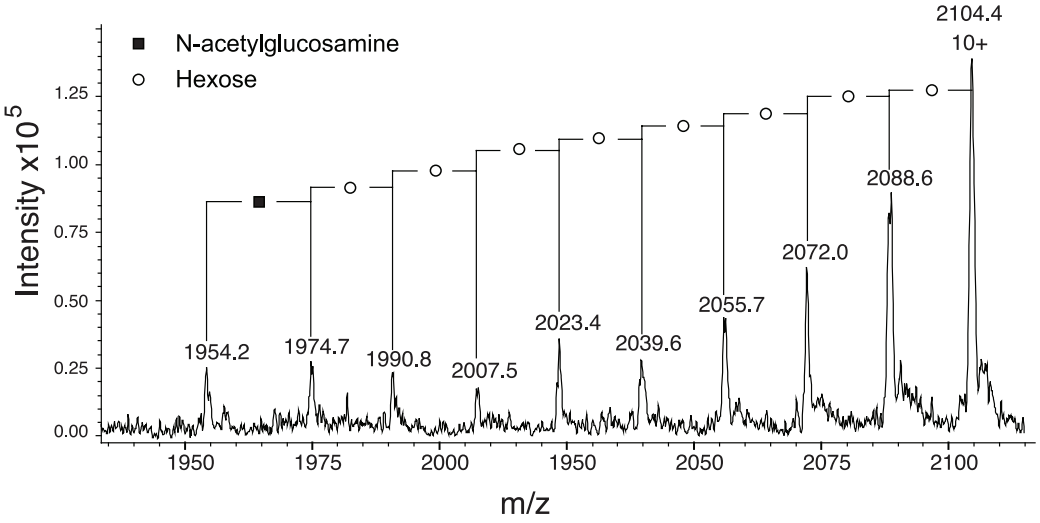


**Table S1 - Statistics of behavioural experiments:** All behavioural experiments are with *Lissotriton helveticus*. Stimuli are as follows: Lh = *Lissotriton helveticus* courtship water, *Lv* = *Lissotriton vulgaris* courtship water, Ia = *Ichthyosaura alpestris* courtship water; CP = *Lissotriton helveticus* courtship-specific peak, MF = non-courting male-female water; SPF-A = SPF 1 + SP 3 glycoforms; SPF-B = SPF 3 glycoforms only. N = the number of couples tested. f indicates the number of couples in which following behaviour was observed for at least 30 seconds.

| **Kruskal-Wallis** | **stimulus 1** | N | f | **stimulus 2** | N | f | **Mann-Whitney U** | *U* | *Z* |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Species-specificity** | | | | | |
| | Lh1 | 12 | 9 | Control 1 | 11 | 1 | **<0.01** | *23.000* | *-2.647* |
| **<0.001** | Lh1 | 12 | 9 | Lv | 12 | 4 | 0.065 | *40.000* | *-1.848* |
| | Lh1 | 12 | 9 | Ia | 12 | 0 | **<0.01** | *15.000* | *-3.291* |
| | | | | **Courtship peak** | | | | | |
| | CP | 10 | 9 | Control 2 | 12 | 1 | **<0.05** | *25.000* | *-2.308* |
| **<0.001** | CP | 10 | 9 | Lh2 | 13 | 11 | 0.385 | *51.000* | *-0.868* |
| | MF | 11 | 2 | Control 2 | 12 | 1 | 0.166 | *43.500* | *-1.386* |
| | | | | **Purified SPF** | | | | | |
| | SPF-A | 10 | 8 | Control 3 | 11 | 0 | **<0.01** | *14.000* | *-2.888* |
| **<0.01** | | | | | | | | | |
| | SPF-B | 10 | 5 | Control 3 | 11 | 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. Meassured 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 |
| Crowngroup Plethodontidae | 8 | 70.6 | 41.7 - 101.2 |
| *L. vulgaris* vs *L. montandoni* | 9 | 16.3 | 7.2 - 27.0 |
| *L. vulgaris* vs *L. montandoni* | 10 | 13.2 | 6.1 - 21.2 |