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5 # Ancient Pheromone Blend as an Alternative for Copulation
6 # in Internally Fertilizing Salamanders
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30 Keywords: evolution, phylogeny, gene duplications, amphibians, protein pheromones
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32 **Throughout the animal kingdom, internal fertilization - the merging of sperm and egg**
33 **inside the female body - nearly invariably relies on the use of a copulatory organ. In**
34 **contrast, males of advanced salamanders (Salamandroidea) attain internal fertilization**
35 **by depositing a spermatophore on the substrate in the environment, which females**
36 **subsequently take up with their cloaca. The aquatically reproducing modern Eurasian**
37 **newts (Salamandridae) have taken this to extremes, since the majority does not display**
38 **close physical contact between the sexes and largely rely on females following the male**
39 **track at spermatophore deposition. Although pheromones have been widely assumed to**
40 **form an important aspect of their courtship, molecules able to induce the female**
41 **following behaviour that culminates in insemination have not been identified. Here we**
42 **show that uncleaved glycosylated SPF protein pheromones, secreted during courtship,**
43 **are sufficient to elicit such behaviour in palmate newts (*Lissotriton h. helveticus*),**
44 **indicating that these molecules obviate the need for copulation in these salamanders.**
45 **Surprisingly, our finding of side-by-side secretion of Late Palaeozoic diverged proteins**
46 **in a single species suggests that these molecules already had a courtship function in stem**
47 **salamanders about 300 million years ago, rendering them one of the oldest vertebrate**
48 **pheromone systems.**
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1. Introduction

Internal fertilization (i.e., the merging of sperm and egg inside the female body) is a widespread reproductive mode that is generally accomplished through copulation, i.e. the insertion of a copulatory organ into the female sex organ (Beckett 1986). In contrast, males of most advanced salamanders (Salamandroidea, making up about 90 % of the more than 650 species of extant salamanders) reproduce by internal fertilization, but deposit a sperm package (spermatophore) on the substrate in the environment, which females subsequently take up with their cloaca. In most families, an enhanced success rate of insemination is accomplished through contact, such as a coordinated tail-straddling walk, or amplexus in which the male sometimes drags the female over the spermatophore (Halliday 1990, Houck et al. 2003). However, some male salamanders have abandoned close physical contact altogether and instead largely rely on tail-fanning courtship pheromones to the female (Halliday 1990, Sever et al. 1990, Verrell et al. 1986). These pheromones induce following behaviour, which is a prerequisite for the subsequent behaviour (e.g., the male displaying a "break" position, the female touching the male's tail) that culminates in insemination (figure 1a, see supplementary movie S1 for the typical behavioural sequence of the courtship process) (Treer et al. 2013, Wambreuse and Bels 1984, Secondi et al. 2005).

In Caudata, the use of protein pheromones during male courtship rituals is known from terrestrial plethodontid salamanders, and a decapeptide attractant in Asian newts has been intensively studied (Cummins and Bowie 2012, Houck et al. 2008, Iwata et al. 2004, Kikuyama and Toyoda 1999, Kikuyama et al. 1995, Osikowski et al. 2008, Palmer et al. 2007, Woodley 2010). However, no studies are available that have characterized pheromones that directly affect the female following behaviour that is a necessary prerequisite for attaining insemination in most aquatically reproducing newts. Here we isolated and purified courtship proteins that are tail-fanned by palmate newts (*Lissotriton helveticus*, Salamandridae) directly from the water, experimentally tested them, and used transcriptomics and phylogenetics to estimate the age of the earliest divergence of present-day secreted proteins.

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80 ## 2. Results and Discussion

81

82 *Salamandrid courtship pheromones progressively evolve towards species-specificity*

83 We first optimized a behavioural assay in which female courtship responses can be
84 measured in the absence of a male, thus giving experimental control over the application of
85 candidate pheromones. A two-female experiment **(**see material and methods) with palmate
86 newts (*Lissotriton h. helveticus*) was used in which females are exposed to male molecules
87 emitted during tail-fanning (figure 1a). Under natural conditions, females respond to male
88 courtship pheromones by following the male closely for a prolonged period (supplementary
89 movie S1). This female following behaviour is a necessary prerequisite to allow other
90 courtship responses leading to successful insemination. The set-up of our two-female
91 behavioural assay removes the secondary sexual morphological and chemical characteristics,
92 as well as the visual cues of tail-fanning of a male, while retaining the required presence of
93 another individual necessary to exhibit following behaviour. Using this assay, we first
94 measured whether water in which a male had been tail-fanning (henceforth termed courtship
95 water) was able to induce such following behaviour in females. As a first indication, we
96 quantified following behaviour by counting the number of couples in which a trained observer
97 measured more than half a minute of following behaviour during ten minutes of observation
98 (henceforth termed Nf). However, to have a fully objective way of measuring female
99 courtship responses, statistical comparisons - a Kruskal-Wallis test (KW) followed by a *post*
100 *hoc* two-tailed Mann-Whitney U test (MW) - were performed with pointing behaviour (i.e.
101 the time period that females faced each other during the experiment, which is largely caused
102 by following behaviour, see Materials and Methods). Our behavioural assays indicate that
103 palmate newt courtship water induced following of conspecific females (figure 1b, species
104 specificity; Lh1: Nf = 9/12, compared to negative control 1: Nf = 1/11; Pointing: P_{KW} <
105 *0.001,* $P_{MW} < 0.01$, table S1). These tests confirm that courtship water is able to induce female
106 courtship responses in palmate newts, even in the absence of the male secondary sexual
107 characteristics and visual cues associated with tail-fanning. Females of palmate newts show a
108 reduced response in courtship water of the congeneric species *L. vulgaris* (figure 1b, species
109 specificity; Lv: Nf = 4/12; Pointing: $P_{KW} < 0.001$, $P_{MW} = 0.065$, table S1). Hybridization and
110 introgression between both species can occur in sympatric ponds (Johanet et al., 2011) and
111 may partially explain these observations. However, female palmate newts showed no
112 following behaviour in courtship water of the more distantly related alpine newts

113 (*Ichthyosaura alpestris*) (figure 1b species specificity, Ia: Nf = 0/12; Pointing: $P_{KW} < 0.001$,
114 $P_{MW} < 0.01$, table S1), suggesting that tail-fanned courtship pheromones progressively evolve
115 towards species-specificity.
116
117
118 *Uncleaved glycosylated SPF proteins induce female courtship behaviour in palmate newts*
119 We optimized a protocol for sampling proteins emitted during male tail-fanning
120 directly from water and compared reversed-phase high-performance liquid chromatography
121 (RP-HPLC) elution profiles of courtship water with that of water in which a non-courting
122 male and female had been held. These analyses show a recurrent pattern of elution profiles
123 showing a peak that is present in water in which a male has been tail-fanning (>20 profiles,
124 one shown; figure 1c, courting couple, courtship peak in orange), but absent in (*i*) water in
125 which non-courting males and females were held for the same amount of time (5 profiles, one
126 shown; figure 1c, non-courting male-female, blue) and (*ii*) courtship water sampled at the end
127 of the breeding season (supplementary figure S1). A behavioural assay with the RP-HPLC
128 fractions of the courtship peak (CP, figure 1c, in orange) indicated that they induced
129 following (figure 1b, courtship specificity; CP: Nf = 9/10, compared to water, negative
130 control 2: Nf = 1/12; Pointing: $P_{KW} < 0.001$, $P_{MW} = < 0.05$, table S1) in a way that is not
131 significantly different from that observed in courtship water (figure 1b, courtship specificity;
132 Lh2: Nf = 11/13; Pointing: $P_{KW} < 0.001$, $P_{MW} = 0.385$, table S1). Conversely, RP-HPLC
133 fractions of non-courting male-female water (MF, figure 1c, blue) resulted in female reactions
134 that were not significantly different from those in control water (figure 1b, courtship
135 specificity; MF: Nf = 2/11; control 2: Nf = 1/12; Pointing: $P_{KW} < 0.001$, $P_{MW} = 0.166$, table
136 S1). These observations indicate that the RP-HPLC fractions of the courtship peak effectively
137 contain the male courtship pheromones that can induce female following behaviour. N-
138 terminal amino acid sequencing (Edman sequencing) of these pooled fractions indicated the
139 presence of multiple isoforms of the Sodefrin Precursor-like Factor (SPF) family (see
140 supplementary table S2, Supplementary Material online). These proteins were considered
141 ideal pheromone candidates, because a full-length protein (i.e., not cleaved, except for the
142 signal peptide) of this family identified from the mental gland of the terrestrially reproducing
143 plethodontid salamander *Desmognathus ocoee* was shown to increase female receptivity
144 (Houck, Watts, Mead, Palmer, Arnold, Feldhoff and Feldhoff 2008).

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145 We further characterized the diversity of SPF proteins by combining transcriptome
146 analyses of the pheromone-producing, sexually dimorphic abdominal gland of a single male
147 with proteome analyses of RP-HPLC fractions of the courtship peak. Whole transcriptome
148 sequencing (RNAseq) of this male gland and de novo assembly of nearly 52 million (Mio)
149 reads revealed 4.1 Mio reads (7.9 %) belonging to this SPF family of molecules. RACE-PCR
150 sequencing revealed 32 different cDNA precursor sequences (GenBank numbers KJ402326 -
151 KJ402357) encoding for 31 unique mature proteins. RNAseq expression analyses indicated
152 five isoforms as most abundant, together making up 94.0% of the SPF transcripts identified in
153 the transcriptome (figure 2a, 2b). Interestingly, the pairwise amino acid divergences between
154 these sequences were between 19.2 and 78.8 %, indicating that these proteins do not only
155 result from allelic variation.

156 To determine the presence of post-translational modifications and to confirm that these
157 precursors are also effectively translated and tail-fanned to the female, we performed an RP-
158 HPLC with a prolonged gradient (figure 2c) to obtain a better separation of the SPF proteins,
159 and combined mass spectrometry analyses with Edman sequencing of individual fractions.
160 Mass spectrometry analyses indicated the presence of an oligosaccharide with 2 N-
161 acetylglucosamine units (GlcNAc) and multiple hexoses (see supplementary figure S2)
162 attached to the available glycosylation sites of the proteins. Individual RP-HPLC fractions
163 revealed the presence of multiple proteins for which the glycosylated masses (up to eight
164 hexoses) match the theoretically predicted masses derived from the five most abundant cDNA
165 precursors (see supplementary table S3, Supplementary Material online). Additionally,
166 several of these predictions could be confirmed by N- terminal amino acid sequencing (see
167 supplementary table S2, Supplementary Material online). This indicates that SPF is
168 effectively present as multiple uncleaved (i.e., except for the signal peptide) proteins with
169 different levels of glycosylation (glycoforms) in the courtship peak.

170 Next we performed ion exchange chromatography to purify SPF from the courtship
171 peak fractions. SDS-PAGE, mass spectrometry and Edman sequencing all indicated that this
172 led to removal of non-SPF as well as some of the SPF proteins, and resulted in a sample
173 containing two SPF proteins (SPF 1: Mr = 21036.8; SPF 3: Mr = 20326.9) with multiple
174 glycoforms (figure 2d). A two-female behavioural experiment with these proteins resulted in
175 a significant increase of the female following behaviour compared to control water (SPF A:
176 Nf = 8/10; control 3: Nf = 0/11; Pointing: $P_{KW} < 0.01$, $P_{MW} < 0.01$, table S1; figure 2d), and
177 confirms that SPF proteins alone are able to induce female following, even in the absence of
178 visual stimuli of a courting male. Finally, we used the same techniques to purify a single SPF

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179 isoform (SPF 3: Mr = 20326.9) with its glycoforms (figure 2e). A behavioural test with this
180 protein induced following in half of the couples compared to none in control water (SPF B:
181 Nf = 5/10; control 3: Nf = 0/11; Pointing: $P_{KW} < 0.01$, $P_{MW} < 0.05$, table S1; figure 2e). This
182 experiment indicates that a single isoform is able to elicit female courtship responses. Future
183 investigations on the relative ability of various individual SPF proteins to induce female
184 following in the palmate newt and related species could give important insights in the
185 evolution of species-specificity of protein pheromones.
186
187
188 *Side-by-side secreted proteins originated from a Late Palaeozoic gene duplication*
189 Phylogenetic analyses combining our palmate newt SPF cDNA precursors with the
190 available sequences on Genbank confirm that SPF diversification goes beyond allelic
191 variation by indicating multiple gene duplication events (figure 3). Speciation-duplication
192 analyses identified speciation events that conform to established higher-level phylogenetic
193 relationships of salamanders (figure 3a), but also recognized two duplications (figure 3b,
194 nodes 1 and 2) that occurred before the Plethodontidae-Salamandridae divergence (figure 3b,
195 node 3). The strongly supported relationship of SPF proteins from lungless salamanders
196 (Plethodontidae) with a clade uniting an *Ambystoma* and our salamandrid SPF3 precursors
197 (figure 3b, indicated with an asterisk) reveals orthologs corresponding to the Salamandridae-
198 Plethodontidae divergence (Hedges et al. 2006, Pyron 2010, Roelants et al. 2007, Vietes et al.
199 2007, Wiens 2007, Zhang and Wake 2009), and defines a split (figure 3B, node 3) that had
200 remained unidentified in previous SPF studies (Cummins and Bowie 2012, Houck et al. 2008,
201 Iwata et al. 2004, Kikuyama and Toyoda 1999, Kikuyama et al. 1995, Osikowski, et al. 2008,
202 Palmer et al. 2007, Woodley 2010). We used the mean and standard deviation (175.7 +/-14.8
203 Mya) of the last five studies presented on Timetree (version of 13 January 2014) to calibrate
204 this node with a central 95% range of 146.7-204.7 Mya, and estimated precursor divergence
205 times with a Bayesian relaxed molecular clock model implemented in Beast (Drummond et al.
206 2012). Our results reveal a Late Palaeozoic gene duplication event (figure 3b, node 1) that
207 denotes the early onset of SPF diversification and secretion (the latter as indicated by our
208 protein characterization from courtship water) at about 288.4 Mya (95% HPD = 200.6-385.1
209 mya) (see supplementary table S4). Our time estimates for salamander speciation nodes in the
210 gene tree (supplementary table S4) are close to the mean of the last five studies (Pyron 2010,
211 Roelants et al. 2007, Vietes et al. 2007, Wiens 2007, Zhang and Wake 2009) in Timetree

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212 (Hedges et al., 2006) that estimated the divergence times for both the Ambystomatidae-
213 Salamandridae split (this study: 143.4 MYA; Timetree: 146.8 Mya) and the onset of
214 diversification of Plethodontidae (this study: 70.6 MYA; Timetree: 72.1 Mya). Additionally,
215 the two nodes that represent the same speciation event in *Lissotriton* in our gene tree (figure
216 3b, nodes 9 and 10, *L. vulgaris* versus *L. montandoni*) have similar age estimates (16.3 MYA
217 and 13.2 MYA, respectively). All these results together strengthen confidence in our
218 divergence time estimates, including the duplication events of SPF genes. The timing of the
219 earliest SPF divergence therefore is close to the origin of stem salamanders (Anura-Caudata
220 divergence, figure 3, estimated at 295.5 +/- 21 Mya from the mean of the last five studies
221 (Pyron 2010, Pyron 2011, San Mauro 2010 , Vietes et al. 2007, Zhang and Wake 2009) in
222 Timetree (Hedges et al. 2006) and considerably predates the currently known use of this
223 protein system (i.e., in crowngroup plethodontids, figure 3, green circle and branches).
224 Our study not only characterizes the pheromones behind the intriguing female
225 following behaviour in salamandrid newts, but also expands the evidence for the use of
226 uncleaved SPF pheromones from a single family (Plethodontidae) to potentially all
227 salamanders. Uncleaved SPF proteins until now were shown to be functional as a pheromone
228 in a single plethodontid species *Desmognathus ocoee* (figure 3b, green circle) (Houck et al.
229 2008). Although an SPF-derived pheromone was initially discovered in a salamandrid, the
230 cleaved active decapeptide (sodefrin, an attractant in *Cynops*) originated through a
231 translational frame shift and as a consequence shows no homology with uncleaved SPF
232 protein pheromones. Additionally, the short peptide obtained its pheromone function in the
233 genus *Cynops*, and therefore independently from uncleaved proteins (Janssenswillen et al.,
234 submitted). To our knowledge, our study of an aquatic salamandrid is the first to expand the
235 effective behavioural evidence for a courtship pheromone function of uncleaved SPF proteins
236 outside the family of plethodontids. Additionally, the side-by-side secretion of anciently
237 diverged proteins (figure 3b, red circles) in our newt species suggests that the courtship
238 function for these proteins considerably predates the Salamandridae-Plethodontidae
239 divergence. Although cDNA studies in individual species already indicated the presence of
240 multiple isoforms, the known diversity of SPF precursors in each of these families resulted
241 from family-specific gene duplications and/or polymorphisms (Osikowski et al. 2008, Palmer
242 et al. 2007). In contrast, our palmate newts tail-fan proteins (figure 3b red circles) of which
243 the estimated divergence dates back to the Late Palaeozoic (figure 3b, node 1) and our results
244 therefore strongly suggest a pheromone function for these molecules already in the earliest
245 salamanders, about 300 mya.

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246 Our combined evidence indicates that, although very different courtship behaviours
247 can be observed across the evolutionary tree of salamanders (Houck et al. 2003, Houck et al.
248 2008, Kikuyama et al. 1995), the function of uncleaved SPF proteins to regulate female
249 sexual receptiveness originated early in salamander evolution and has been conserved with
250 various observable effects in multiple salamander lineages ever since. In palmate newts, and
251 likely also in related species with female following behaviour, these pheromones obviate the
252 copulatory organ by stimulating the female following behaviour that is a prerequisite for
253 successful fertilization.
254
255

256 3. Material and methods

257

258 (a) Animals

259 The research was done with permission and according to the guidelines of Agentschap
260 voor Natuur en Bos (permits ANB/BL-FF/V12-00050 and ANB/BL-FF/V13-00134). All
261 experiments complied with EU and Belgian regulations concerning animal welfare. The
262 catching method and the housing conditions were the same to those described elsewhere
263 (Treer et al. 2013). Animals were released back to the pond of their origin after the
264 experiments were finished. We used 40 adult males and 40 females of each of the three
265 species of newts (*Lissotriton helveticus*, *L. vulgaris* and *Ichthyosaura alpestris*). All species
266 have an overlapping breeding season and were collected in spring from ponds near Ternat,
267 Belgium.
268

269 (b) Behavioural experiments

270 *Receptivity tests*
271 To evaluate suitability of the animals to be used, all experiments were preceded by a
272 receptivity test before the first experiment. For this test, each female was placed together with
273 a male, and only females that showed following behaviour were selected for subsequent
274 (positive and control) two-female tests (Treer et al. 2013). For the species-specificity
275 experiments, *L. vulgaris* and *I. alpestris* females were tested in parallel to confirm the potency
276 of their courtship water used to test *L. helveticus* females. For detailed description of the
277 receptivity tests, see elsewhere (Treer et al. 2013).
278

279 *Stimuli*

280 Tests were done with (*i*) courtship water of *L. helveticus, L. vulgaris,* or *I. alpestris,* or (*ii*)
281 aged tap water containing purified proteins (RP-HPLC and ion exchange fractions) of *L.*
282 *helveticus*. The aged tap water had been kept in the room where the animals were housed,
283 ensuring similar temperature conditions. Collection of courtship water was done by putting a
284 receptive male and a female in a plastic container (25x16x14 cm) filled with 800 ml of aged
285 tap water. We measured twenty minutes of cumulative male tail-fanning duration as a
286 standard. The couple was then removed and courtship water was immediately used. For more
287 information on the purified proteins from courting couples and male-female water see
288 purification of proteins and Results and discussion. Control behavioural experiments were
289 done in aged tap water.

290

291 *Two-female test*

292 We conducted two-female behavioural experiments modified from (Treer et al. 2013) using *L.*
293 *h. helveticus* females. Treer *et al.* showed that two alpine newt females that were kept in water
294 in which a conspecific male had been tail fanning showed natural courtship responses such as
295 following and tail touching in absence of a male. With this notion, female courtship responses
296 can be measured in absence of a male, having complete control over the application of
297 candidate pheromones. Female-female interactions have been reported for several species
298 during courtship (e.g., Waights 1996). Although it cannot be excluded that females not only
299 physically interact but also use chemical cues, this would not influence our results, since our
300 statistics are based on differences between positive (chemical cue present) and negative
301 (control) waters, while female pheromones would be present in both samples.

302 Behavioural experiments with two *L. h. helveticus* females were done in 600 ml aged
303 tap water with and without stimuli added, or in 600 ml courtship water (see stimuli section).
304 All experiments were performed on consecutive days, at the same time of the day, and under
305 the same light and temperature conditions (see Treer et al. 2013 for more details). In all tests,
306 female behaviour was recorded for twelve minutes, and the first two minutes of the
307 experiment were discarded to allow acclimatization of the animals. Experiments were
308 recorded using a digital camera connected directly to a computer, and the recordings were
309 analysed. First, we counted the females that showed interest towards one another, which
310 includes turning towards the other female and following her (following behaviour). Females
311 showing more than 30 seconds of following behaviour were scored as positive (Nf). This
312 following behaviour is female courtship behaviour similar to that under natural conditions

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313 with a courting male, where a female starts to closely follow his movements (Wambreuse and
314 Bels 1984). Second, to have an objective measurement that could be used in statistical
315 comparisons we calculated the cumulative amount of time females pointed towards each
316 other. Pointing is a way to measure the change in behaviour without having to understand or
317 observe specific types of female behaviour and is therefore objective. We measured the
318 cumulative amount of time (seconds) per couple that an imaginary straightforward line,
319 perpendicular to the line connecting the eyes of the following female, intersects the other
320 female's body. Although unintended pointing is also measured this way, such random noise
321 occurs in both the stimulus-containing water and the blank, and any significant difference
322 between both experiments is therefore caused by pheromone-induced following behaviour.
323 The differences in pointing between stimuli were tested with the Kruskal-Wallis test followed
324 by post hoc two-tailed Mann-Whitney U test for pairwise comparisons (Dytham 2011). The
325 analyses were done using IBM SPSS Statistics for Windows (IBM SPSS Statistics for
326 Windows Version 22.0. IBM Corp, Armonk, NY, 2013).
327

328 **(c) Collection and extraction of molecules from courting couples water and male-female**
329 **water**

330 Protein collection was done by placing a male and female for one hour in a plastic
331 container (25x16x14 cm) filled with 600 ml of water. Male-female couples were monitored
332 for courtship behaviour and the amount of time a male fanned his tail was measured. For each
333 condition, we sampled a minimum of 15 courting couples in which at least ten minutes of
334 male tail-fanning occurred. The water of a non-courting couples (male-female water, no tail-
335 fanning) was collected in the same way and for the same amount of time as above, but for
336 each animal separately to ensure that there was no courtship display. Molecules were
337 extracted by applying non-courtship or courtship water of a single couple onto two separate
338 solid phase extraction cartridges (300 ml per filter; RP-C8 and RP-C18 Sep-Pak plus
339 cartridge, 400 mg sorbent, Waters, Milford, MA, USA) using a vacuum pump. Proteins were
340 eluted from both cartridges with 7.5 ml of 90% (v/v) acetonitrile containing 0.1% (v/v) TFA.
341 All acetonitrile was evaporated using a SpeedVac concentrator (SCV-100H, Savant
342 instruments, Farmingdale, NY) for 1 h. After concentration, samples were pooled per
343 condition and subjected to RP-HPLC (see Purification of proteins).
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345 **(d) Purification of proteins**

346 Peptides and proteins were partially separated using reversed-phase high-performance
347 liquid chromatography (RP-HPLC). Pooled and concentrated samples (*L. helveticus* courtship
348 water or male-female (non-courtship) water) were loaded onto a Source 5RPC column
349 (4.6x150 mm, GE Healthcare Life Sciences, Uppsala, Sweden) pre-equilibrated with 0.1%
350 (v/v) TFA (A). After loading, the column was washed for 10 minutes at a constant flow rate
351 of 1 ml/min using the same solvent. Proteins were eluted with 80% acetonitrile in 0.1% TFA
352 (B) by applying a linear (from 0-100 % B in 80 minutes at 1 ml/min) or flattened gradient
353 (30-65% B in 56 minutes at 1 ml/min). Detection of eluting proteins was performed at a
354 wavelength of 214 nm and the eluate was collected in fractions of 1 ml. Fractions of interest
355 were subjected to non-reducing SDS-PAGE using precast gels (Any kD Mini-PROTEAN
356 TGX, Biorad, Hercules, CA, USA). Proteins were visualized by silver staining (Silverquest
357 Silver Staining kit, Invitrogen, Carlsbad, CA, USA). For subsequent behavioural tests with
358 fractions of interest (figure 1c, blue and orange), the acetonitrile and TFA was evaporated
359 using a SpeedVac concentrator (SCV-100H, Savant instruments, Farmingdale, NY).

360 To further purify the candidate pheromones, HPLC fractions of interest from the
361 courtship water sample were submitted to ion exchange chromatography (see figure 2D and
362 2E). After evaporating the acetonitrile (SCV-100H, Savant instruments, Farmingdale, NY)
363 samples were brought to pH of 7.5 by addition of buffer containing 20 mM bis-tris propane,
364 20 mM piperazine and 20 mM N-methyl piperazine (Sigma). Samples were loaded onto a 1
365 ml Hitrap DEAE Fast Flow (GE Healtcare Bio-sciences, flow rate 1ml/min) column pre-
366 equilibrated with binding buffer containing 15 mM bis-tris propane, 15 mM piperazine and 15
367 mM N-methyl piperazine (buffer A, pH 7.5, Sigma) and washed for at least 10 minutes with
368 the same buffer until all material in the effluent disappeared. Proteins were eluted with 15
369 mM bis-tris propane, 15 mM piperazine and 15 mM N-methyl piperazine (buffer B, pH 3,
370 Sigma) by applying a linear gradient (from 0-100% B in 20 minutes). Detection of eluting
371 proteins was performed at a wavelength of 280 nm and the eluate was collected in fractions of
372 1 ml. Purity of the fractions was assessed by mass spectrometry and non-reducing SDS-
373 PAGE, using precast gels (Any kD Mini-PROTEAN TGX, Biorad, Hercules, CA, USA).
374 After electrophoretic separation, proteins were visualized by silver staining (Silverquest
375 Silver Staining kit, Invitrogen, Carlsbad, CA, USA) and fractions of interest were used in 2-
376 female tests (fractions A and B in figure 2D and 2E).

377

378

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378 **(e) Mass spectrometry and amino acid sequence analyses**

379 Mass analyses of the HPLC fractions were performed by electrospray ionization ion
380 trap mass spectrometry on an ESQUIRE- LC MS (Bruker, Brussels, Belgium). In addition
381 mass analyses of the desalted ion exchange fractions (Zip Tip C18, 10 µl, Millipore) were
382 performed on an Amazon Speed ETD ion trap mass spectrometer. Characterization of the
383 glycan moiety was done through in-source fragmentation on the Esquire ion trap mass
384 spectrometer by gradually elevating the potential on skimmer 1 and the exit caps in the
385 electrospray source. Peak fractions of courtship water collected during breeding season were
386 subjected to a non-reducing SDS-PAGE using precast gels (Any kD Mini-PROTEAN TGX,
387 BioRad, Hercules, CA, USA). After electrophoresis, proteins were transferred from the gel
388 onto a PVDF membrane by semi-dry blotting (Trans Blot Turbo System, Bio-Rad) and
389 stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA; membrane
390 not shown). All protein bands were excised from the blot for N-terminal sequencing on a 491
391 Procise cLC protein sequencer (Applied Biosystems, Foster City, CA, USA).
392

393 **(f) Transcriptomics and gene expression estimates**

394 The sexually dimorphic abdominal glands for RNA sequencing (RNA-seq) were
395 sampled from a single male. Total RNA was extracted using TRI Reagent (Sigma-Aldrich)
396 and the RNAeasy mini kit (Qiagen). Extracted RNA was sent to Baseclear (Leiden, The
397 Netherlands) for RNA sequencing, de novo transcriptome assembly, and gene expression
398 estimations. A pair-end cDNA sequencing library (PE50) was created with Illumina TruSeq
399 RNA Library Preparation Kit and 52040842 fragments were sequenced on an Illumina HiSeq
400 2500 instrument. FastQ reads were generated after analyses with Illumina Casava pipeline
401 (version 1.8.3), a post-filtering script (Baseclear) and FASTQC quality control tool (version
402 0.10.0) to remove low quality, PhiX-control and adapter reads. De novo transcriptome
403 assembly was performed with Trinity (Grabherr et al. 2011) and transcript expression levels
404 were estimated by mapping reads to the de novo assembled transcripts, on the RNA-seq
405 module of the CLC Genomics Workbench (allowing two mismatches per read). SPF
406 sequences were identified through aligning assembled transcripts to a dataset containing SPF
407 sequences from the Uniprot database using RAPsearch (Ye et al. 2011).

408 RACE (rapid amplification of cDNA ends) was performed to obtain complete protein
409 sequences from different SPF precursors. Primers were designed on the 3’-untranslated region
410 to amplify full-coding sequences of SPF transcripts as follows:

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411 SPF_Primer_A, 5'-TTGTTAATAAWYATTCTGTAAAGARGCT-3'; SPF_Primer_B, 5'-
412 GCCTTGTTGBCAAAAHKTCTTC-3'; SPF_Primer_C, 5' ACAAYTWCTAAGCTGGHKTAGGA-3';
413 SPF_Primer_D, 5'-GTGTGTATWTGRGGTATRAACAAAGGTC-3', SPF_Primer_E, 5'-
414 CCAACAATTACTRRGMKGGAGTAGG-3'; SPF_Primer_F, 5'-CAACTACTAAGCTRRAGTM
415 RGAGTGC-3'; SPF_Primer_G, 5'-GGRTAGGATTGCGTCAGATGTT-3'; SPF_Primer_H, 5'-
416 TAGGAATGTTTCTAYKGACKACTACTRAG-3'; SPF_Primer_I, 5'-CTATTGCTAAGCTG
417 KGGTG-3'; SPF_Primer_J, 5'-GCTGGCACATGGGCATGT-3'; SPF_Primer_K, 5'-GCCCAWA
418 CASKACTAAGCACATT-3'; SPF_Primer_L, 5'-GACTCTGVATTHCAGGTACTTGTAGAG-3'.
419 A total of 1 μg total RNA from the same extraction procedure as in RNA-seq was used to
420 create RACE cDNA with the SMARTer-RACE cDNA amplification kit (Clontech). PCR
421 products were amplified with FastStart High Fidelity Taq DNA polymerase (Roche).
422 Amplification products were cloned into a pGEM-T Easy cloning vector (Promega) and
423 vectors were transformed into TOP10 Competent Cells (Invitrogen). Colonies were picked
424 randomly and inserts were amplified with Faststart Taq DNA polymerase. Amplification
425 products were purified and sequenced by the VIB genetic service facility (Antwerp, Belgium).
426 For comparison with protein masses found in courtship water, contiguous sequences (contigs)
427 were assembled with CodonCode Aligner 3.7.1.1 (CodonCode Corporation) using a 99%
428 similarity threshold, after quality trimming. Signal peptides, predicted using SignalP 4.0
429 (Petersen et al. 2011), were removed and protein masses were calculated with the pI/Mw tool
430 on Expasy (http://web.expasy.org/compute_pi/). Gene expression differences between SPF
431 homologs were estimated using RNA-seq read counts (as described above) on the assembled
432 homologs from our RACE procedure. Expression levels were determined using RPKM
433 (Reads Per Kilobase per Million mapped reads) values (Mortazavi et al. 2008).
434

435 **(g) Phylogeny and divergence time estimates**

436 We combined protein sequences of a representative set of 16 SPF precursors of
437 *Lissotriton helveticus* found in this study with available sequences (representing the major
438 currently available evolutionary lineages) of SPF precursors of four plethodontid (GenBank
439 nrs AAZ06338, AAZ06329, AAZ06335, AAZ06331), three ambystomatid (GenBank nrs
440 CN040015, CN041146, CN048649) and six other salamandrid sequences (i.e. two *Lissotriton*
441 *montandoni*, GenBank nrs ACB54670 and ACB54672, and four *Lissotriton vulgaris*,
442 GenBank nrs ACB54665, ACB54666, ACB54668 and ACB54669). Two frog sequences
443 (GenBank nrs XP_002943341, F6PQG9) were chosen as outgroup. Alignment of the protein

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444 sequences was done with MAFFT (Katoh and Standley 2013) using the L-INS-i method
445 (automatically assigned) and resulted in a data matrix of 216 amino acids. Maximum
446 Likelihood (ML) analyses were run in PAUP (Swofford), using a LG amino-acid rate matrix,
447 with empirical frequencies, estimated proportion of invariable sites (0.0527345) and
448 distribution of rates at variable sites following a gamma distribution with four categories and
449 estimated shape parameter (1.79548). This resulted in a single ML tree with likelihood score
450 (-Ln L = 8271.589). Bayesian analyses and Bayesian posterior probabilities were calculated in
451 MrBayes (Ronquist et al. 2012). Two runs of four Markov chain Monte Carlo (MCMC)
452 chains each were executed in parallel for 5,000,000 generations, with a sampling interval of
453 500 generations and a burn-in corresponding to the first 1,000,000 generations. Convergence
454 of the parallel runs was confirmed by split frequency standard deviations (< 0.01) and
455 potential scale reduction factors (approximating 1.0) for all model parameters. Adequate
456 posterior sampling was verified using Tracer 1.5 (Rambaut et al. 2014), by checking if the
457 runs had reached effective sampling sizes >200 for all model parameters. Speciation-
458 duplication analyses were done using Notung (Chen et al. 2000). To estimate the age of the
459 earliest diversification in our SPF pheromones, we used a Bayesian relaxed molecular clock
460 model (uncorrelated Log-normal) implemented in Beast (Drummond et al. 2012) that
461 accounts for lineage-specific rate heterogeneity. As a calibration point, we used the
462 divergence of the (Salamandridae, Ambystomatidae) clade from Plethodontidae, a
463 relationship that is widely accepted (Pyron 2010, Roelants et al. 2007, Vietes et al. 2007,
464 Wiens 2007, Zhang and Wake 2009) and was also recovered in our ML tree. We used the
465 mean (175.7 Mya) and standard deviation (14.8) of the last five studies (Pyron 2010, Roelants
466 et al. 2007, Vietes et al. 2007, Wiens 2007, Zhang and Wake 2009) presented on Timetree
467 (Hedges et al. 2006) (version of 13 January 2014) to calibrate this node in our tree with a
468 central 95% range of 146.7-204.7 Mya (calculated in Beast). Posterior sampling was verified
469 using Tracer 1.5 (Rambaut et al. 2014). The sodefrin precursor sequence of *Cynops* was not
470 included, because the end of the sequence (containing the sodefrin peptide) is not homologous
471 with our full-length proteins.
472

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472 **Acknowledgments**

473

474 We thank Dirk Cieters and Luc Platteau from Natuurpunt Ternat, Dimitri Dufour and the
475 Vermeir family for their collaboration and help in obtaining the newts for the research. We
476 thank Han Remaut and Ghislain Opdenakker for use of lab equipment, Rik Janssens and
477 Mieke Gouwy for lab assistance, and Willem Meilink for drawing salamander figures. We
478 also thank the Agentschap voor Natuur en Bos for giving permits. This research was
479 supported by a European Research Council starting grant [ERC 204509, project TAPAS], the
480 Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen [grant no. G.0133.08] and the
481 Hercules foundation (purchase of LC-MS equipment, AKUL/11/31). SJ receives a grant from
482 the IWT, MM receives an aspirant fellowship from FWO, IVB, PK and AM are supported by
483 a postdoctoral fellowship from FWO.

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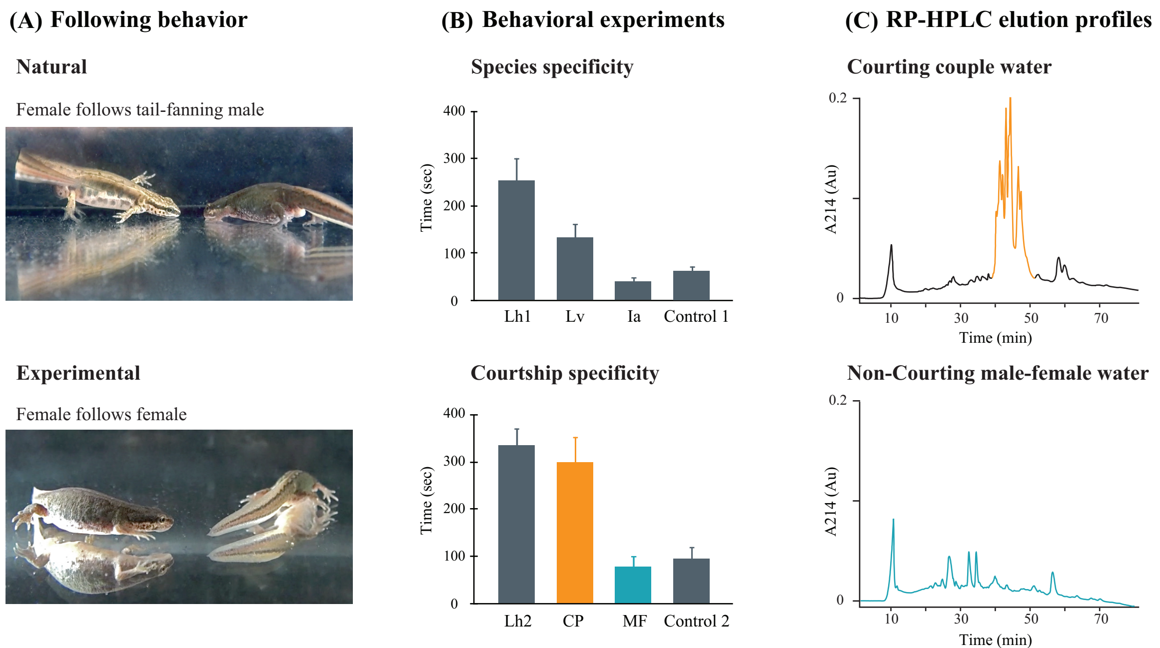
484 **Figures**

485



486

487

488 **Figure 1. Identification and isolation of *L. helveticus* male courtship pheromones.**
489 (**A**) Up: Tail-fanning of the pheromones towards the nose of the female persuades her to
490 follow the path of the retreating male; Down: analogous following response of a female in
491 pheromone-containing water during a two-female behavioural bio-assay. (**B**) Behavioural
492 assays Up: Species-specificity 2-female tests showing the mean cumulative duration (+/−
493 S.E.) of *L. helveticus* females' pointing behaviour in courtship water of: their own species
494 (Lh1, N=12), *L. vulgaris* (Lv, N=12), *I. alpestris* (Ia, N=12) and negative control (Control 1,
495 H_2O, N=11); Down: Courtship specificity 2-female tests in *L. helveticus* showing the mean
496 cumulative duration (+/− S.E.) of *L. helveticus* females' pointing behaviour in: courtship
497 water (Lh2, N=13), RP-HPLC fractions composing the courtship peak (CP, orange, N=10),
498 RP-HPLC fractions of a non-courting couple (MF, blue, N=11) and negative control (Control
499 2, H_2O, N=12). N is the number of couples tested (**C**) Comparison of RP-HPLC profiles of
500 courtship water and male-female (non-courtship) water. Courtship water shows a courtship
501 peak (orange) that is absent in MF water (blue), indicating that males largely release courtship
502 pheromones during tail-fanning (that induce female following). Coloured fractions were
503 pooled and used in the 2-female courtship specificity tests.

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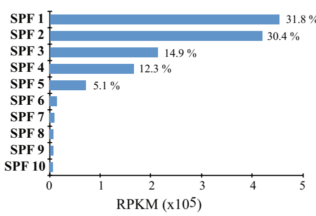

(B) Most abundant SPF precursors

Signal peptide

```
SPF 1 MRAFLAAVVMLQALI-TGDC LLCEKCLVSGTTQCSGIFKQCPPDVTHCVKGLENNTL-GDTVILTAFKDCMDPSEQAACGKGFQFQNSGFNLQISRTCCDSDFCNK
SPF 2 MKAFLAAVVMLQALI-TGDC LLCETCLASGTSQCSGIFKQCSPDVTHCVKGLENGTL-GGNVILTVFKDCLDPSQQVACGREFQFQNSRYFIQISRTCCDSDFCNK
SPF 3 MMSFAASASLFLAFIATGNA IECEVCSNRASLDCSGDLVTCDQSVESCQTAIAEYSFEGLDPIYNVFKNCSDVRSKNTLYRSAE-KHGSYYLRVE--VCKTNGCNK
SPF 4 MRAFLAAVAMFQALI-TGDC LLCEQCFTTGTAQCSGIFKQCSPDVTHCVKGIENSTL-GSEVTLTAFKDCLDPSHKATCGRKSSLKTSVLSLIISRTCCDSDFCNG
SPF 5 MKAFLAAVVMLQALI-TGDC ILCEKCLATSTTQCSGIFKQCSPDVTHCVKGLENNTL-GDTVVVTAFKDCMNPSQQAACGREFHFQNSGFSLQISRTCCDSDFCNK

                                                                                                              Mr
SPF 1 GEVEVPAVDQTPNGYKCDECLTDQSS-GCTPTGGIQCTGEQNTCSTFYGKALRPGIILEPYSMKACATPDFCEIYYPVATLVFSYELFCAPAKKQ   19331.8
SPF 2 GEVEVPAVDQTPNGYICDECLTQQSSEACTPTGQAHCTGKQNTCSSFYGSALRTGGTLRSYSMKACATQDSCDLYFPVATVFYGYHSQCVPAQKL   19448.7
SPF 3 SPLEFPPKNTALNGVRCPTCSVDGDL-SCEATEILDCVGEMTSCIYHAAQFRVSAAPAVSAAFRGCTSAGSREQLPVTPADTIEDVVTAIISKGV   18945.2
SPF 4 GDIQVPAVDEIPNGYKCEDCFTSQSADTCTEAAEVQCTGEHNTCLSFTGTGSRPGEGVYHYTVKGCVTQDYCQLFYLVGTQTYTYDLQCSPAKKL   19358.6
SPF 5 GEVEVPAVDETPNGYMCDECLTDQSSESCTPTGHVHCTGVQNTCSTFYGSAIIPGGTMQPYSMKGCTTPDYCEIYYLVATLVDSYELRCVPAQKQ   19557.8
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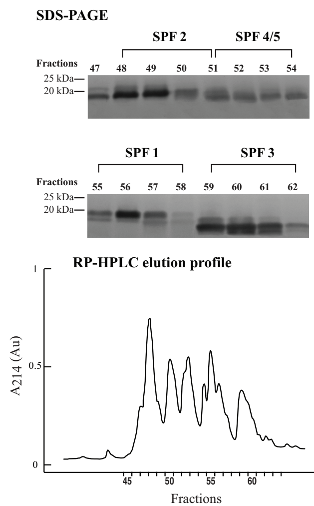
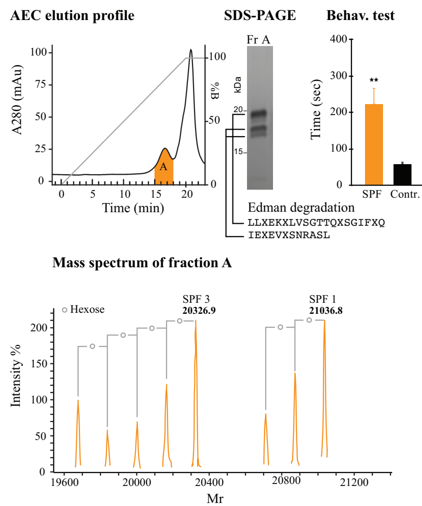
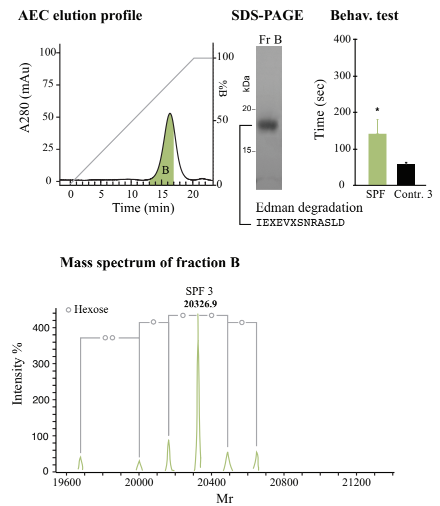






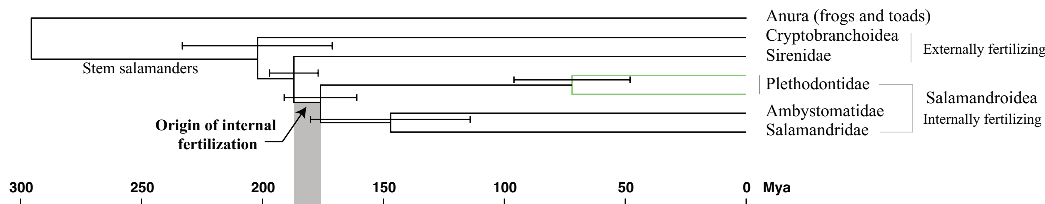
505

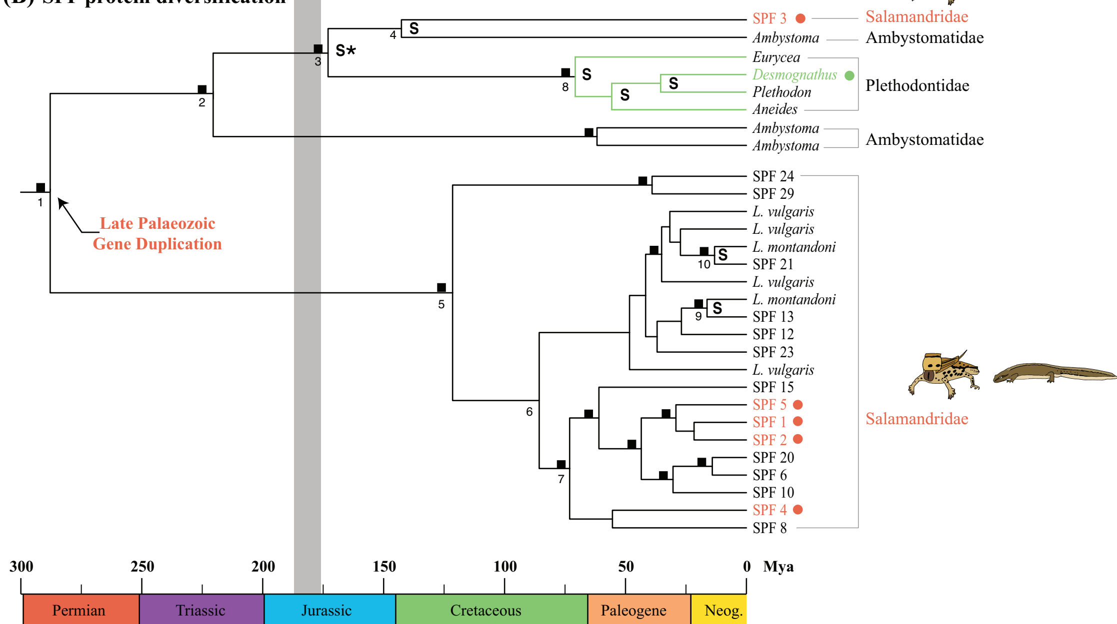
506

507 **Figure 2. Transcriptomic and proteomic analyses of SPF proteins.** (**A**) RNAseq
508 expression level (percentage of total SPF) of the ten most abundant SPF precursors in the
509 abdominal gland of a male (Reads Per Kilobase per Million mapped reads, RPKM). (**B**)
510 MAFFT alignment and theoretical masses of the five most abundant SPF proteins found in
511 the abdominal gland. Cysteins are indicated in grey. (**C**) SDS-PAGE (silver staining) and RP-
512 HPLC elution profile of SPF proteins in a courtship peak. Our analyses show that the SPF
513 proteins present in the courtship water match the five most abundant RNA precursors found
514 in the abdominal gland. See supplementary table S2 for Edman sequencing and table S3 for
515 mass spectrometry analysis of individual fractions. (**D, E**) Anion exchange chromatography
516 (AEC) elution profile, silver stained SDS-PAGE, mass spectrometry (deconvoluted mass
517 spectra), Edman sequencing, and behavioural tests of two SPF pheromones (**D**) and a single
518 SPF pheromone (**E)** Asterisks indicate significance levels: *P < 0.05, **P < 0.01.

519

520





520

521 **Figure 3. Time estimates (A) species diversification**. The mean and standard
522 deviations for species diversifications were calculated from the last five published estimates
523 (Pyron 2010, Roelants et al. 2007, Vietes et al. 2007, Wiens 2007, Zhang and Wake 2009).
524 The origin of internal fertilization in the ancestor of Salamandroidea is indicated in gray. **(B)**
525 **SPF protein diversification.** The tree shows Bayesian dating estimates, the asterisk denotes
526 the calibration point. The diversity of plethodontid precursors was chosen to reflect the largest
527 known SPF divergences in this family. The fact that our gene tree of plethodontid SPF's
528 corresponds to the higher taxonomic level relationships of these species therefore indicates
529 that the known SPF variation is the result of family-specific variation and/or gene
530 duplications (in agreement with Palmer, et al. 2007). Bayesian Posterior Probabilities >95 are
531 indicated with black squares. Speciation nodes are indicated with S, all other nodes are
532 considered duplication nodes (by Notung analyses, see Materials and methods). Node
533 numbers refer to age estimates in table S4. Numbered SPF's all indicate sequences from the
534 abdominal gland of the palmate newt *L. helveticus*. The top five expressed proteins, which
535 were also confirmed in courtship water, are indicated with red circles. The green circle
536 denotes the species in which the pheromone function was demonstrated in plethodontids.

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