Frog nuptial pads secrete mating season-specific proteins related to salamander pheromones

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Short title: Frog nuptial pad proteins

Summary

Males of many frog species develop spiny nuptial pads with underlying glands on their thumbs during the mating period. We used 3D visualisation on the European common frog *Rana temporaria* to show that these glands' morphology allows the channeling of secreted molecules to the pad's surface during amplexus. Combined transcriptome and proteome analyses show that proteins of the Ly-6/uPAR family, here termed amplexins, are highly expressed in the nuptial glands during the mating season, but are totally absent outside that period. The function of amplexins remains unknown, but it is interesting to note that they share structural similarities with Plethodontid Modulating Factors, proteins that influence courtship duration in salamanders.

1. INTRODUCTION

As with many animal taxa, amphibians are expected to make extensive use of chemical communication during courtship and reproduction. In salamanders (Urodela), several courtship pheromones have already been characterized, and most of them turned out to be proteins or peptides (Houck, 2009). Frogs and toads (Anura) make extensive use of acoustic signals during the mating period, and it has therefore long been assumed that chemical communication was more limited in this group. During the past two decades, however, increasing evidence from behavioural experiments has shown chemical communication to be more widespread among anuran taxa than previously assumed (Houck, 2009), but the molecules influencing anuran behaviour remain largely uncharacterized.

During the mating season, males of most species of frogs and toads can often be observed taking a piggyback ride on the female (Figure 1A). This so-called amplexus is necessary for coordinating egg deposition and sperm release during the typical anuran process of external fertilization (Duellman and Trueb, 1986). During the annual mating season, male frogs develop keratinized, often spiny nuptial pads on their thumbs and forearms. It is generally acknowledged that these pads serve to improve the male's grip on the female during amplexus (Duellman and Trueb, 1986). Histological studies additionally have shown the presence of glands below the surface of the nuptial pads (Thomas et al., 1993). Because these glands release their secretion

only onto the keratinized surface of nuptial pads (Kyriakopoulou-sklavounou et al., 2012), it has been proposed that they produce glue-like substances to enhance the male's grip on the female (Brizzi et al., 2003). However, nuptial and other sexually dimorphic skin glands (SDSGs) differ histochemically from other anuran skin glands and share features with known pheromone glands in salamanders, being both multicellular, alveolar glands with a granular secretion product (Thomas et al., 1993). This indicates that nuptial pads may also synthesize chemical signals involved in courtship and mating. Moreover, pheromones produced in the mental glands of some plethodontid salamanders are delivered transdermally (Houck and Reagan, 1990). This is particularly interesting, because inspection of *Rana* females directly after egg laying shows that the ventral skin is often abraded at the site where the male's spiny nuptial pads have been holding the female (Figure 1B), leaving the possibility of a similar way of delivering chemical signals in anurans.

We used micro-CT scan imaging of the nuptial pad of the European common frog *Rana temporaria* Linneaus (1758), to show that the nuptial gland morphology allows the channelling of secreted molecules to the pad's surface. We subsequently screened the transcriptome of the nuptial glands for candidate pheromones or other proteins with a possible signalling function by construction of a cDNA library and we compared the proteome of the nuptial pad in the breeding and non-breeding seasons.

2. MATERIAL AND METHODS

Micro-CT scanning

Collection of animals was done with permit ANB/BL-FF/V11-00033. Sampling complied with EU and Belgian regulations concerning animal welfare. The thumb of a male specimen of *Rana temporaria* (Haacht, Belgium; 29/03/11; sampled on day of capture) was fixed in 4% formalin. To visualize soft tissue organization using X-ray tomography, a postfixation in a 1% solution of osmium-tetroxide was performed for five hours (a common postfixation for electron microscopy) (Labor Impex, Brussels, Belgium). The thumb was subsequently scanned at the UGCT scanning facility at Ghent University using a transmission-type micro-focus X-ray tube (FeinFocus FXE160.51). The tube voltage was set to 80 kV and tube current was set to 112 µA, providing sufficiently small spot size. Specimens were mounted on a controllable rotating table (MICOS, UPR160F-AIR). Covering 360°, a series of 1440 projections of 2008x1778 pixels was recorded using a PerkinElmer XRD 1620 CN3 CS flat-panel

detector. A geometric magnification of 54 was achieved, resulting in an isotropic reconstructed voxel size of 3.7 µm. Reconstruction of the tomographic projection data was done using the in-house developed Octopus-package. The 3D volume rendering of the reconstructed sections was done using Amira 5.4.0 (Visage Imaging Inc., Berlin, Germany), where individual glands were manually segmented.

Histology

The thumbs of two male specimens (Haacht, Belgium; captured on 29/03/11 and sampled on the same day) were removed and fixed in 4% formalin. The thumb pads with surrounding skin was surgically removed and embedded in paraffin (Histosec, 56-58°C, Merck Belgium). Sections of 5 and 7 µm were cut using a Prosan Microm HM360 microtome equipped with disposable metal knives. Alternating sections were stained with an improved trichrome staining (for general histological details of the tissues) (Mangakis et al., 1964) or with a Periodic Acid Schiff reagens (to stain the mucus of the integumental glands) (Carson and Hladik, 2009). Sections were subsequently mounted on glass slides using DPX (VWR International, Belgium) and imaged using a Zeiss Polyvar microscope equipped with a Colorview8 digital camera.

cDNA Library

A cDNA library was constructed from the two nuptial pads of a single individual (Haacht, Belgium; 29/03/11; sampled on day of capture) during the mating season. RNA was extracted with TRI reagent, using manufacturer instructions (Sigma-Aldrich, Bornem, Belgium). A 0.05 µg of total RNA was reverse transcribed and cloned into a vector using the Creator SMART cDNA library construction kit (Clontech, Leusden, The Netherlands). Transformation was performed with One Shot® TOP10 Electrocomp™ *E. coli* electrocompetent cells (Invitrogen, Ghent, Belgium) and colonies were grown on LB agar plates containing chloramphenicol (30µg/ml final concentration). Colonies were picked randomly and amplified using vector specific primers (M13). The following PCR conditions were used: one initial denaturation for 240s at 94°C, followed by 25 cycles with denaturation for 40s at 94°C annealing for 60s at 55°C, and elongation for 60s at 72°C. Amplification products were purified with a PCR-purification kit (Qiagen, Hilden, Germany) and 571 clones were sequenced on an ABI Prism 3100 Genetic Analyzer (Applied

Biosystems, Halle, Belgium). CodonCode Aligner 3.7.1.1 (CodonCode Corporation, Centreville, Massachusetts, USA) was used for base calling, vector and quality clipping and assemblage of contiguous sequences (contigs). The mRNA sequences were translated into their corresponding AA-sequence using the Expasy translating tool (http://web.expasy.org/translate/) and molecular mass was calculated using the software Sequence Editor (Bruker, Brussels, Belgium). BLAST (Basic Local Alignment Search Tool) was used to compare the nucleotide sequences to the nucleotide database of the National Center for Biotechnology Information (blastn) and the translated nucleotide sequences (all reading frames) were compared to the protein database (blastx).

RACE PCR

We performed 3′-RACE (rapid amplification of cDNA ends) PCR to obtain full-length sequences of mRNA molecules of interest using the SMARTer-RACE cDNA amplification kit (Clontech, Leusden, The Netherlands). Molecules of interest were selected based on transcript abundance in the cDNA library, presence of a signal peptide and similarities with known vertebrate pheromones. The cDNA was reverse-transcribed from 1 µg total RNA extracted from the nuptial pad of one individual male (Haacht, Belgium; 29/03/11; sampled on day of capture and stored in RNA-later). One gene specific primer designed on the signal peptide region of the molecule of interest (GCAGAACATCANRATGAAAGC) was used to amplify the 3’ side of the mRNA transcript. The following PCR conditions were used: one initial denaturation for 240s at 94°C, followed by 36 cycles with denaturation for 40s at 94°C, annealing for 60s at 60°C, and elongation for 60s at 72°C. Amplification products were cloned using a pGEM-T Easy cloning vector (Promega, Leiden, The Netherlands) and vectors were transformed into DH5α™ Competent Cells (Invitrogen, Ghent, Belgium). Seventy-two colonies were picked randomly and inserts were amplified using the same PCR conditions as described above. Amplification products were purified using the Wizard SV 96 PCR Clean-Up System (Promega, Leiden, The Netherlands). Purified products were cycle-sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and visualized on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Halle, Belgium). Sequence editing and contig assembly was performed with CodonCode Aligner 3.7.1.1 (CodonCode Corporation, Centreville, Massachusetts, USA). Using the MAFFT online server (http://mafft.cbrc.jp/alignment/software/), sequences of

interest were aligned with Plethodontid Modulating Factor (PMF) sequences (*Plethodon shermani,* AEO22663.1 and *Aneides ferreus*, ABI48851.1), a salamander courtship pheromone of the Ly-6/uPAR protein family (Palmer et al., 2007).

HPLC

To compare protein content in breeding and non-breeding seasons, we surgically removed nuptial pads from males several times during the year (breeding: 15/03/11, Haacht, Belgium; non breeding: 18/06/10, Haacht, Belgium, 3/11/10, Brugge, Belgium and 2/08/11, Haacht, Belgium; all samples were taken on the same day of frog collection) and placed them in 1 ml Amphibian Ringer Solution (ARS) – 0.8 mM Acetylcholine chloride (AcChCl) for 30 min (4°C). Each sample was subsequently centrifuged (4°C – 15 min - 14000 rpm) and the supernatant was filtered through an Ultrafree-MC 0.22 µm spin down filter (Millipore, Overijse, Belgium). Samples were dried using a Univapo 150 ECH vacuum concentrator connected to a FTS VT490 Cold Trap and Edwards RV3 vacuum pump. They were then resolved in 2% acetonitrile (CH3CN) with 0.1% trifluoroacetic acid (TFA) and subsequently loaded on a Beckman System Gold High Performance Liquid Chromatographer equipped with a diode array detector 168 and programmable solvent module 126. We used a Waters Symmetry C8 column (5 µm; 4.6 x 250 mm). After an initial five minutes of washing with 98% solvent A (0.1% TFA) and 2% of solvent B (80% CH3CN, 0.1% TFA), concentration of solvent B was linearly increased to 100% in 55 minutes. Flow rate was 1 ml/min and fractions were collected every minute using an automated Gilson fraction collector 202. Alternatively, some samples were loaded on a Source 5 RPC column (4.6 x 150 mm) in 0.1% TFA and eluted with a linear CH3CN gradient (0% to 80%) in 0.1% TFA on a Waters 600 HPLC system. UV absorbance of the eluted proteins was detected at 214 nm and part (1/150) of the effluent was split on-line to an ion trap mass spectrometer. Fractions were stored at – 20°C.

Mass analyses

Mass analyses of the HPLC fractions were performed by electrospray ionization ion trap mass spectrometry on an ESQUIRE-LC MS (Bruker, Brussels, Belgium). In addition, stored fractions were analysed on an Ultraflex II MALDI TOF/TOF mass spectrometer (Bruker, Brussels, Belgium). Each fraction was lyophilized and resuspended in 100 µl milliQ H_2O. One µl was mixed on the metal target with an

equal volume of matrix solution (50 mM α-cyano 4-hydroxycinnamic acid in 30% acetonitrile containing 0.1% TFA). The solution was air-dried and introduced into the MALDI TOF/TOF mass spectrometer source. Intensity graphs of mass-to-charge (m/z) were presented through the software FlexAnalysis (Bruker, Brussels, Belgium). Fractions that contained a peak of interest were sequenced *de novo* by means of Edman degradation on a capillary 491 Procise cLC protein sequencer (Applied Biosystems, Halle, Belgium). Detected masses were compared with theoretical masses predicted from the translated cDNA sequences in Sequence Editor (Bruker, Brussels, Belgium).

3. RESULTS AND DISCUSSION

The nuptial pad of *Rana temporaria* covers the entire preaxial part of the thumb (Figure 1C) in male individuals and is completely absent in females. Micro-CT scans of the nuptial pad (Figure 1D) showed the presence of two types of acinar glands in the dermis (Figure 1E), similar to those found in other species (Brizzi et al., 2003; Thomas et al., 1993). They differ at the level of their overall size and thickness of the epithelial wall, but both have a duct exiting at the epidermal surface, in between the keratinous cones of the pads (Figure 1D & E; pores indicated with white arrows). Such morphology allows molecules synthesized and stored in the nuptial glands to be channelled to the pad's surface during amplexus. The larger glands are less numerous, and are lined by low columnar cells containing granules. Considering the large central lumen, these glands seem to have the capacity to temporarily store secretions. The small glands, on the other hand, are lined by high columnar cells, and are also intensively granular. In these glands, the central lumen is reduced and continues into branching crypts running in between the epithelial cells, so storing of secretions is probably limited. Both gland types showed similar stainings with PAS, so no further details about functionality could be derived at this point.

An SDS page of extracted glands confirms the presence of a wide array of proteins (supplementary figure 1). cDNA library construction and subsequent EST sequencing of the nuptial glands showed that the most abundant protein-coding mRNA sequences (3.3% of the transcriptome) during the breeding season encode three isoforms of a small (104 AA) secretory protein of the Ly-6/uPAR protein family, which we termed amplexin (Figure 2A, amplexin 1-3). RACE-PCR on the nuptial

pads identified transcripts encoding two additional isoforms of this protein (Figure 2A, amplexin 4 and 5) (GenBank Numbers KC282376-KC282380). Secretory Ly-6/uPAR proteins are often involved in the modulation of nicotinic and muscarinic acetylcholine receptors (nAChRs and mAChRs, respectively) to elicit neuronal or muscular physiological responses (Adermann et al., 1999). For example, mammalian PATE proteins comprise a considerable group of secretory Ly-6/uPAR proteins that are mainly found in male reproductive organs. Some of these proteins have been shown to interact with nAChRs, which suggests an involvement in the modulation of neural transmission during reproduction and fertility (Levitin et al., 2008). Human SP10 and SAMP14, for example, are involved in the regulation of the sperm-egg interaction (Kong and Park, 2012), and plethodontid PMF is known to act as a courtship pheromone (Palmer et al., 2007). Interestingly, BLAST searches with the amplexins identified PMF as one of the closest related proteins. Alignment of the amplexin isoforms with PMF clearly shows the similarities in protein domains (Figure 2A), and indicates the possible existence of an ancient pheromone system in amphibians.

HPLC profiles and MS analyses revealed a high prevalence of amplexins during the breeding season (Figure 2B, HPLC fractions 24-26). *De novo* sequencing of one of these fractions yielded an N-terminal sequence of LQXYKGSXTGRPTXSLPVEI, which confirms the match with the transcriptome data. Cysteines were not detected (indicated as X in the sequence) as they were not alkylated prior to the sequence analysis. Since Edman degradation of the complete fraction resulted in a single sequence, all protein peaks within the fraction are likely to share this N-terminal sequence. In combination with the detection of proteins that have a related but different mass (by mass spectrometry), this confirms the presence of several isoforms with an identical N-terminus as detected during transcriptome analyses. Importantly, no amplexins were found in the corresponding HPLC fractions from all samples collected in different months outside the breeding season (Figure 2B), and the complete absence of these molecules was confirmed by mass spectrometry. The nuptial pad gradually regresses in the weeks after breeding, but amplexin content was observed to be more abrupt, with a much lower relative abundance at the end of the breeding season, when the nuptial pad is still clearly present. This suggests that changes in amplexin expression are not merely contributing to the yearly process of nuptial pad recrudescence or regression.

Our combined observations and analyses indicate that amplexins are secreted at the male nuptial pad's spiny surface, probably during amplexus. Given that the spines also cause wounds on the female's chest, we hypothesize that the secreted molecules can seep directly in the female's circulatory system. Most vertebrates use olfactory and vomeronasal signal transduction in chemical communication (Brennan and Zufall, 2006), and such a direct delivery of protein pheromones into the circulatory system is only known from some species of plethodontid salamanders (Houck and Reagan, 1990). Males of several plethodontid species develop hypertrophied premaxillary teeth and a sexually dimorphic mental gland during the breeding season, which they use to rub their pheromones into the female skin (Houck and Reagan, 1990). Since nuptial pads and male-specific breeding glands are common in multiple amphibian families (Duellman and Trueb, 1986), it is possible that pheromone delivery through skin abrasion will prove to be a common theme in amphibian reproduction. A function for amplexins is as yet unknown. However, because mating frogs are far less mobile and more vulnerable to predation than single frogs, an accelerated mating process would have an obvious selective advantage. Although speculative, we hypothesize that nuptial pads may secrete pheromones that reduce the duration of amplexus, a function that would be similar to that of combined protein pheromones in plethodontid salamanders (Houck, 2009).

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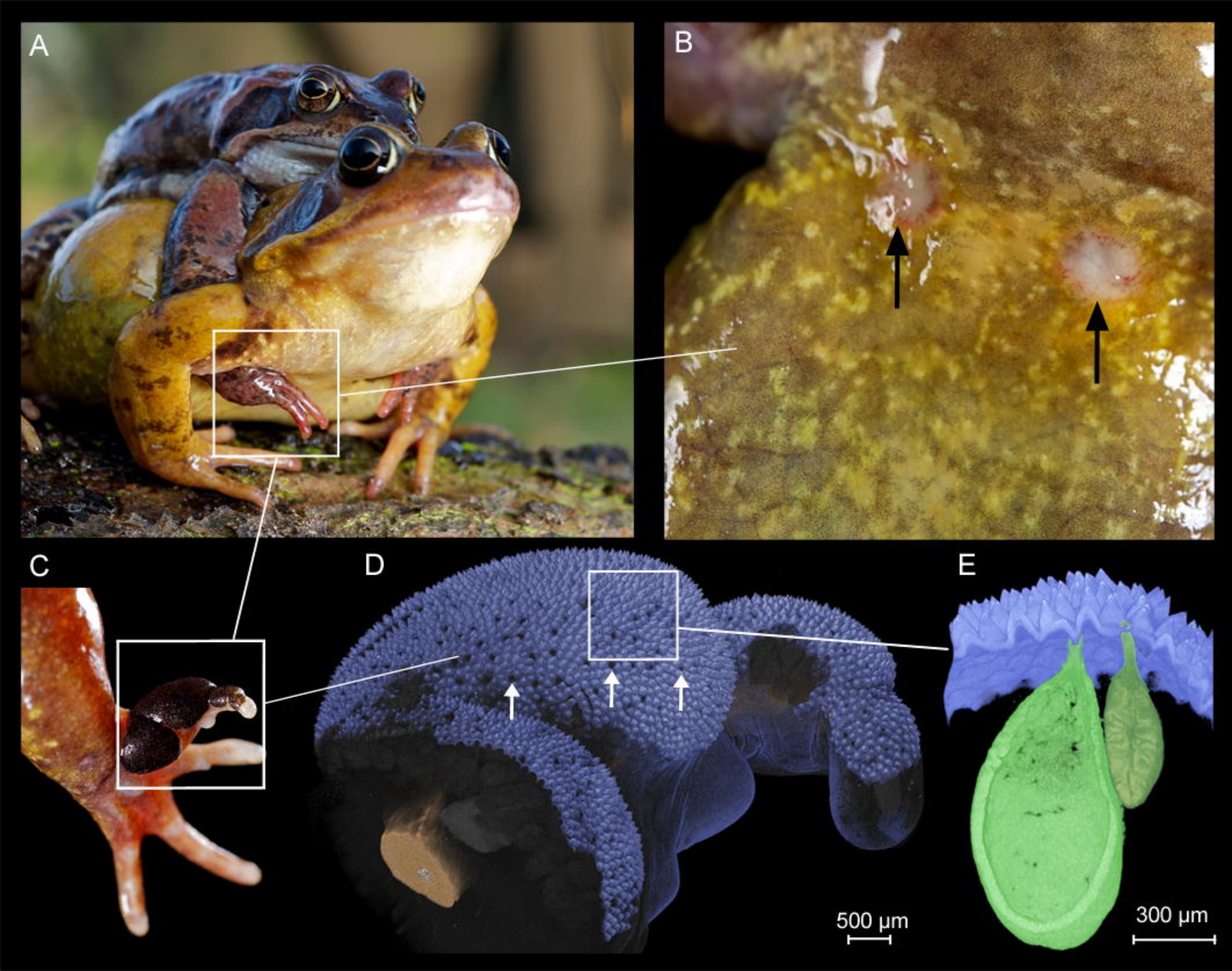
FIGURE LEGENDS

Figure 1. (A) Couple of *Rana temporaria* in amplexus, showing the grip of the male. (B) Wounds (black arrows) on the female chest after amplexus and egg-laying. (C) Nuptial pad on the thumb of a male during the breeding season. (D) 3D visualisation of the thumb of a male, showing the outlet of the glands between the spines (white arrows) at the nuptial pad surface. (E) 3D reconstruction of a cross-section of the nuptial pad, showing two gland types and their channels under the nuptial pad surface.

Figure 2. (A) Alignment of amplexin isoform sequences from the nuptial pad of a frog with PMF-pheromones of two plethodontid salamanders. The conserved cysteins of the Three-Finger Motif are in grey. Asterisks indicate identical amino acids. (B) HPLC spectra of the nuptial pad secretion in breeding (upper spectrum) and non-breeding (lower spectrum) season. The grey window shows the presence (respectively absence) of amplexins in fractions 24 to 26. The grey line shows the linear increase of solvent B.

SUPPLEMENTARY INFORMATION

Supplementary figure 1: (A) SDS page (15%) of the protein content of nuptial glands of a male *Rana temporaria* during the breeding season (Haacht, Belgium; 09/02/10; sampled on day of capture) and (B) PageRuler Plus Prestained Protein ladder (Fermentas) in kilodaltons (kDa). Proteins were extracted using AcChCl and 20 µl of the extracted product was mixed with 5 µl loading buffer and 1 µl DDT for denaturation (5 minutes at room temperature, followed by 3 minutes at 90°C).


A
B
C
D
E
500 µm
300 µm

A

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                    1.......10........20........30........40........50....
                              *       *  * **                *    * *     *
amplexin 1          MKAVISLLFLGLLFLHGEALQCYKGSCTGRPTCSLPVEICTGDQDQCVRRYGIR
amplexin 2          MKAVISLLFLGLLFLHGEALQCYKGSCTGRPTCSLPVEICTGDQDQCVRRTKIR
amplexin 3          MKAVISLLILGLLFLHGEALQCYKGSCTGGPSCSLPVEICTGDQDQCVRRTKIR
amplexin 4          ---------LGLLFLHGEALQCYKGSCTGRPTCSLPVEICTGDQDQCVRRYGIR
amplexin 5          ---------LGLLLLHGEALQCYKGSCTGRPTCSLPVEICTGDQDQCVRRTKIR
PMF Plethodon       MRSAVLLIFLVVFVSTGNSLSCYLKNALED-----GIVTCPTERDNCI---IIK
PMF Aneides         MRATALLVLLVVLVSFGESLKCYYESEGVK-----RIDECNSPDDSCVH--VIS
                    |                   |
                       signal peptide          Three-Finger motif

                    ....60........70........80........90.......100........
                                         *     *                **  ****
amplexin 1          TPKGISANHRGDLTWTTQGCATKANCLELKTIKHYS----RCCSGDLCNSPKEM
amplexin 2          TNTGISANHRGDRAWTTPGCATKANCLELKPMKHHS----RCCSGDLCNSPKEM
amplexin 3          TNTGISANHRGDRAWTTPGCATKANCLELKPMKHHS----RCCSGDLCNSPKEM
amplexin 4          TPKGISANHHGDRAWTTQGCATKATCLELKPMKHHS----RCCSGDLCNAPKEM
amplexin 5          TNTGISANHHGDRAWTTQGCATKATCLELKPMKHHS----RCCSGDLCNAPKEM
PMF Plethodon       TSTRD---------YKA--CASHEFCEKFPELVNDPFEIHRCCQEDLCN-----
PMF Aneides         SKFGV---------WKT--CLLKLFCDDMAEMEKETFPIHVCCTTDLCN-----
                    |____________________________________________________|
                                   Three-Finger motif
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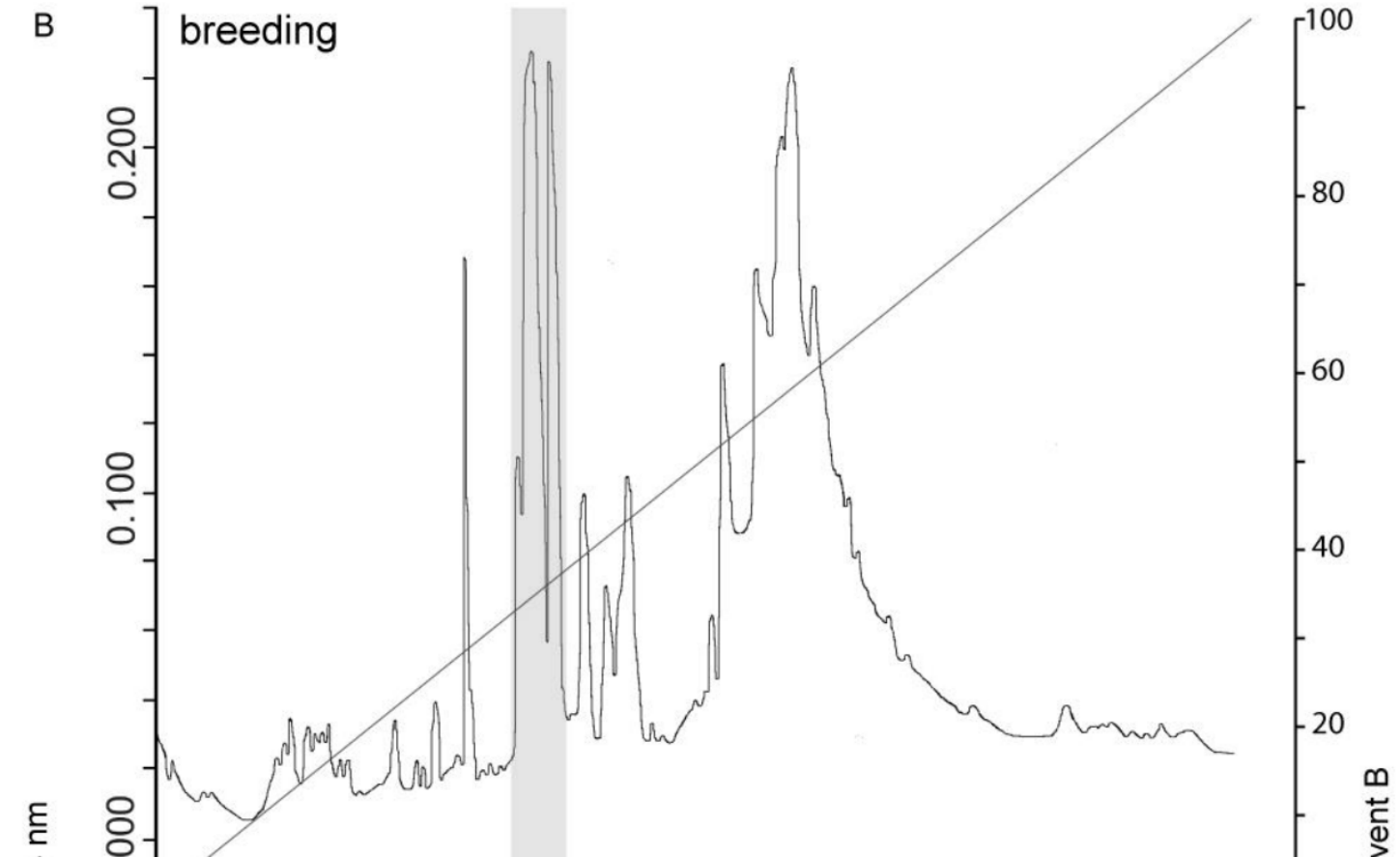
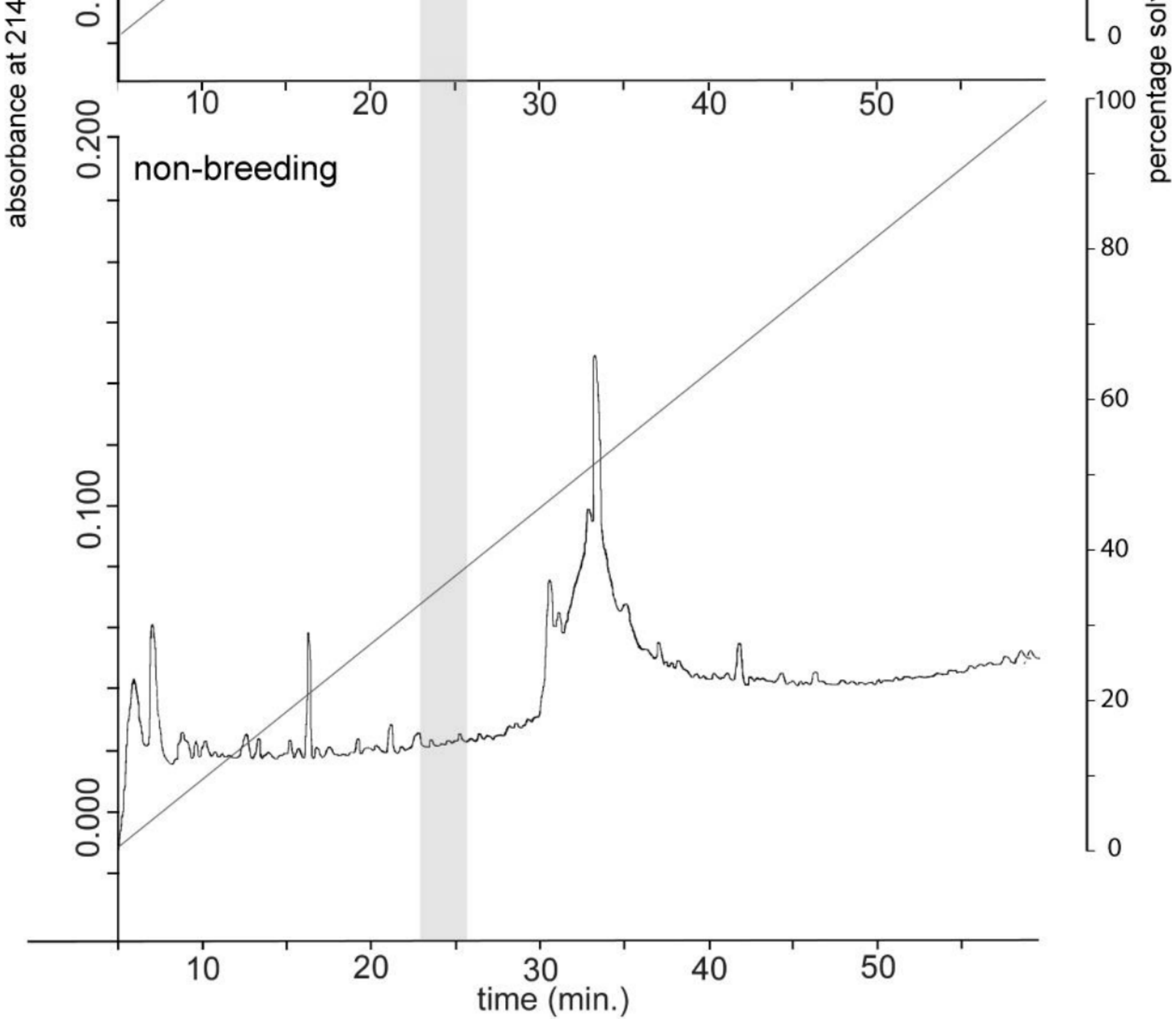
B




non-breeding
absorbance at 214
0.200
0.100
0.000
percentage sol
100
80
60
40
20
0
10
20
30
40
50
time (min.)