

## Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*

### The discovery of the aquatic male sex pheromone splendipherin, together with Phe8 caerulein and a new antibiotic peptide caerin 1.10

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The skin secretions of female and male *Litoria splendida* have been monitored monthly over a three-year period using HPLC and electrospray mass spectrometry. Two minor peptides are present only in the skin secretion of the male. The first of these is the female-attracting aquatic male sex pheromone that we have named splendipherin, a 25 amino acid peptide (GLVSSIGKALGGLLADVVKSGQPA-OH). This pheromone constitutes about 1% of the total skin peptides during the breeding season (January to March), dropping to about 0.1% during the period June to November. Splendipherin attracts the female in water at a concentration of  $10^{-11}$ – $10^{-9}$  M, and is species specific. The second peptide is a wide-spectrum antibiotic of the caerin 1 group, a 25 residue peptide (GLLSVLGSAKHVLPVVPVIAEKL-NH<sub>2</sub>) named caerin 1.10. The neuropeptides of *L. splendida* are also seasonally variable, the change identical for both the female and male. During the period October to March, the sole neuropeptide present in skin secretions is caerulein [pEQDY(SO<sub>3</sub>)TGWMDF-NH<sub>2</sub>]; this is active on smooth muscle and is also an analgaesic. During the southern winter (June to September), more than half of the caerulein is hydrolysed to [pEQDYTGWMDF-NH<sub>2</sub>], a peptide that shows no smooth muscle activity. In place of caerulein, a new peptide, Phe8 caerulein [pEQDY(SO<sub>3</sub>)TGWFDF-NH<sub>2</sub>], becomes a major component of the skin secretion. Perhaps this seasonal change is involved in thermoregulation, that is, with the initiation and maintenance of the inactive (hibernation) phase of the animal.

**Keywords:** antibiotic peptides; male sex pheromone; neuropeptide; splendipherin.

The skins of amphibians are characterized by a rich variety of bio-active peptides (e.g. toxins, neuropeptides and antimicrobials) that form an integral part of the animals' defence systems and also regulate dermal physiological functions [1–3]. In response to a variety of stimuli, the peptides are secreted onto the skin from specialized glands located on the dorsal surface. We have studied the host defence peptides of some 25 species of Australian anuran [4,5]. The Magnificent Tree Frog *Litoria splendida* is a typical example [6–9]: the peptides isolated to date from the combined secretion from the parotoid and rostral glands of this animal are listed in Table 1. Caerulein is a known amphibian neuropeptide [10] with smooth muscle, analgaesic and hormone activity [1] and has been used clinically [1,2]. Caerins 1.1 and 1.6 are wide-spectrum antibiotics, caerins 1.1.1 and 1.1.2 are inactive and are formed by hydrolysis of the major peptide caerin 1.1 by an endoprotease, and caerins 2.1 and 3.1 are narrow spectrum antibiotics. Caeridin 1.1 shows no

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*Abbreviations:* ESMS, electrospray mass spectrometry; MS/MS, collision induced mass spectra.

*Note:* this work conforms with the Code of Practice for the Care and Use of Animals for Scientific Purposes (1990) and the Prevention of Cruelty to Animals Act (1985), and was approved by the University of Adelaide Animal Ethics Committee.

*Note:* this paper is dedicated to the memory of Professor Vittorio Erspamer. (Received 16 September 1999, accepted 12 November 1999)

antibiotic activity: its role in the amphibian integument is not known at this time [4,5].

This paper reports the results of monitoring the glandular secretions of female and male *L. splendida* monthly over a three year period. This program was undertaken for two reasons. First, in our previous studies [6–9] it has seemed that the skin peptide profile of a frog does not vary significantly with the time of the year and, as such, the method may constitute an accurate probe to identify anurans. We wished to investigate this scenario for a particular species of frog and chose *L. splendida* for this purpose because we have already studied its host defence peptides and because we maintain a colony of these frogs in captivity.

The second reason was that we believed that it is possible that anurans have both terrestrial and aquatic pheromones, and we wished to search, in the first instance, for an aquatic sex pheromone. Although pheromones in insects and some animals have been widely studied [11–13], much less is known about the pheromones of amphibians [14]. There has been a report of a suspected alarm pheromone in the toad *Bufo bufo* [15], and the aquatic pheromone sodefrin (SIPSKDALLK-OH) has been isolated from the cloacal gland of the male Japanese Firebellied newt *Cynops pyrrhogaster* [16]. The latter report has encouraged us to initiate a search for pheromones in anurans. We report the first anuran sex pheromone to be discovered and identified, and also that the skin peptide profile of *L. splendida* varies seasonally. A preliminary report of the identification of the sex pheromone has been published [17].

**Table 1.** Major peptides exuded from the parotoid/rostral glands of *L. splendida* [6,9].

Name	Sequence	$M_r$	Activity	Compound no. in Figs 1 and 2
Caerulein	pEQDY(SO <sub>3</sub> )TGWMDF-NH <sub>2</sub>	1352	Neuropeptide	3
Caerin 1.1	GLLSVLGSAKHVLPVVPVIAEHL-NH <sub>2</sub>	2582	Antibiotic	11
Caerin 1.1.1	LSVLGSAKHVLPVVPVIAEHL-NH <sub>2</sub>	2412	Inactive	5
Caerin 1.1.2	SVLGSVAKHVLPVVPVIAEHL-NH <sub>2</sub>	2299	Inactive	4
Caerin 1.6	GLFSVLGAVAKHVLPVVPVIAEKL-NH <sub>2</sub>	2591	Antibiotic	10
Caerin 2.1	GLVSSIGRALGGLLADVVKSKGQPA-OH	2392	Weak antibiotic	8
Caerin 3.1	GLWQKIKDKASELVSGIVEGLK-NH <sub>2</sub>	2382	Antibiotic	7
Caeridin 1.1	GLLDGLLTGL-NH <sub>2</sub>	1140	Inactive	6

## METHODS AND TECHNIQUES

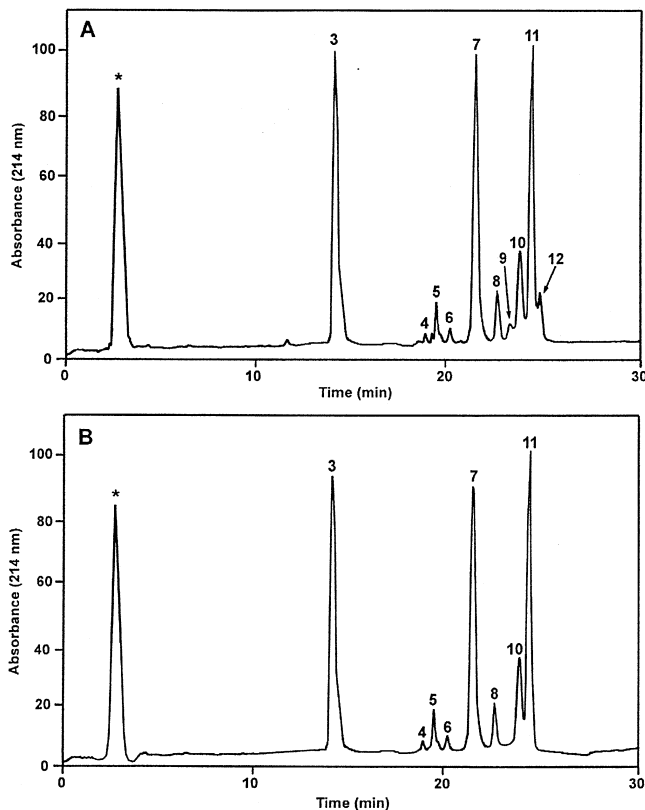
### Preparation of skin secretions

Adult male and female specimens of *L. splendida* were collected from the Kimberley region of Western Australia and maintained in captivity at the University of Adelaide. The temperature of their captive variable environment is maintained at 28 °C with 65% humidity and overhead daylight artificial lighting. Secretions were obtained from parotoid and rostral glands by the non-invasive electrical stimulation method [18]. No animals were sacrificed during these experiments. The skin secretion was washed from the animal with distilled water

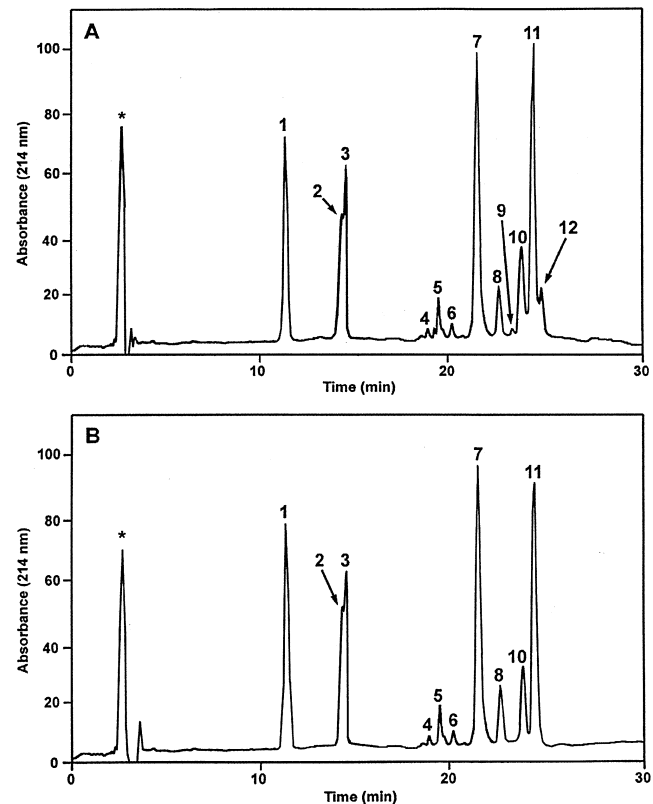
(25 mL). Methanol (25 mL) was immediately added to the aqueous solution, the mixture centrifuged, and concentrated to a volume of 1 mL. Samples were taken from both male and female groups of *L. splendida* monthly over a period of three years and tested by HPLC and electrospray mass spectrometry (ESMS).

### HPLC analysis

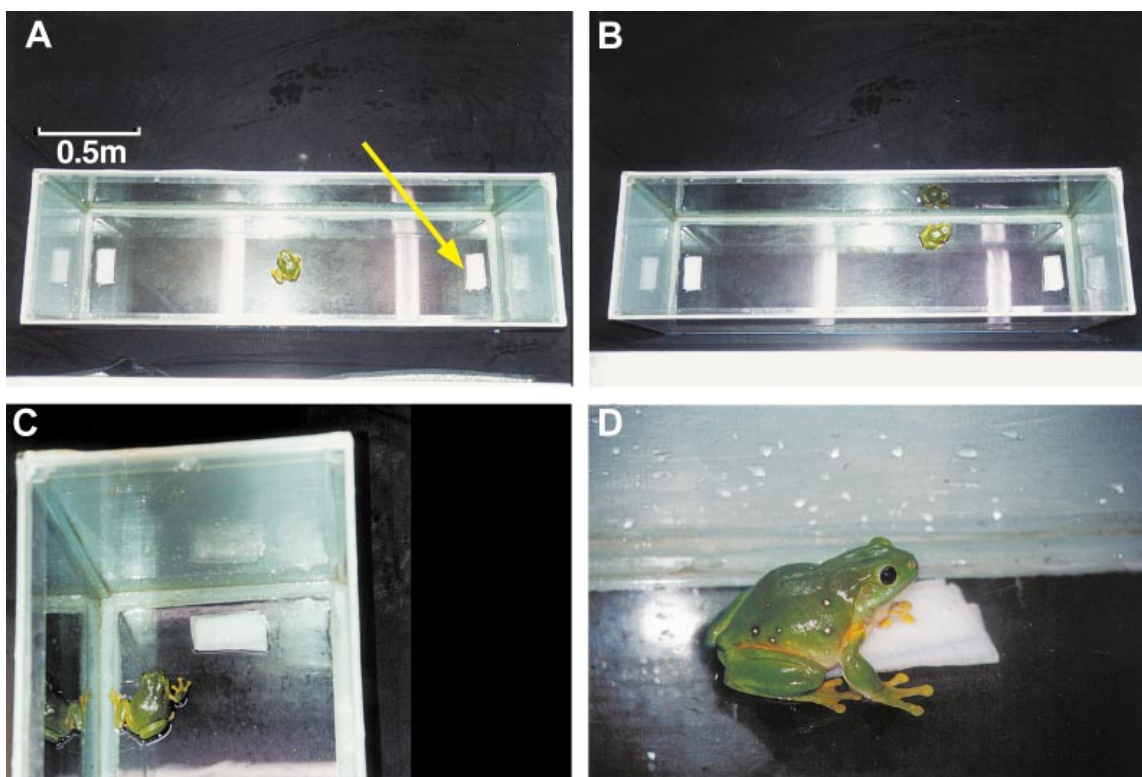
HPLC separation was achieved for each crude secretion using a VYDAC C<sub>18</sub> reverse phase column (5 μm, 300 Å, 4.6 × 250 mm) equilibrated with 10% acetonitrile/aqueous 0.1% trifluoroacetic acid. The elution profile increased from



**Fig. 1.** HPLC of glandular secretions of (A) male and (B) female *L. splendida*, obtained in January 1998. Components 3–8, 10 and 11 have been identified in previous studies [6–9]; their amino acid sequences are listed in Table 1. Components 9 and 12 are splendipherin and caerin 1.10, respectively. \*, Non-peptide material.



**Fig. 2.** HPLC of glandular secretions of (A) male and (B) female *L. splendida*, obtained in July 1998. Components 3–8, 10 and 11 have been identified in previous studies [6–9]; their sequences are listed in Table 1. Compounds 1 and 2 are Phe8 caerulein and desulfated caerulein, respectively; 9 and 12 are splendipherin and caerin 1.10. \*, Non-peptide material.



**Fig. 3.** Aerial view of pheromone behavioural test with female *L. splendida*. (A) Animal seated in the centre of the 2 m tank before pheromone added to swab. Note the two gauze pads (one arrowed) at opposite ends of tank. (B) 3 min after 40 ng of splendipherin added to the gauze pad at the right hand end of the tank. Note that the animal has moved forward towards the top side of the tank. (C) 5 min after addition of pheromone. Female now moving away from the side of the tank directly towards the pheromone source. (D) 7 min after addition of pheromone. Female seated above pheromone source.

10–70% acetonitrile over a period of 40 min using a flow rate of  $1 \text{ cm}^3 \cdot \text{min}^{-1}$ . HPLC profiles are illustrated in Figs 1 and 2.

#### Purification and structure determination of peptides

Crude peptide fractions were separated using HPLC conditions as outlined above. Further HPLC separation of each crude fraction was effected using an elution profile of 40–65% acetonitrile over a period of 60 min giving pure caerulein, desulfated caerulein, Phe8 caerulein, splendipherin and caerin 1.10 following lyophilization. Mass spectral data were determined by ESMS with a Finnigan LCQ ion trap mass spectrometer. The peptide was infused in water/methanol (1 : 1) at  $8 \mu\text{L} \cdot \text{min}^{-1}$ . Collisional activation mass spectral data (MS/MS) were obtained using a collision energy of 35% [9]. The identification of C-terminal  $\text{CO}_2\text{H}$  or  $\text{CONH}_2$  groups was done using ESMS to determine the difference in the masses of the natural peptide and its methyl ester [9]. Automated Edman sequencing was performed by a standard procedure [19] using an applied Biosystem 492A proscribe sequencer equipped with a 900-A data analysis module. Synthetic peptides were prepared by solid-phase chemistry [20] (using all L-amino acids) by Chiron Mimotopes (Clayton, Victoria, Australia).

#### Behavioural studies

Experiments were conducted using a preference test. A glass aquarium ( $65 \times 200 \times 75 \text{ cm}$ ) was filled to a depth of 2 cm with water (1600 mL). Two sterilized cotton gauze pads ( $101 \times 200 \text{ mm}$ ) were folded in half and secured at opposite ends of the tank (Fig. 3). The animal was placed in the centre of the tank and allowed to remain there for 5 min.

Splendipherin [4–4000 ng (as appropriate) in distilled water (0.1 mL)] was added underwater to one of the gauze pads, while the other pad contained only water. All movements of the animal were monitored over a 30-min period. At the conclusion of each experiment the tank was carefully cleaned and dried before the next experiment. Four test females and four males of *L. splendida* were used in single experiments. Similar behavioural studies were also carried out using: a synthetically modified splendipherin (C-terminal  $\text{CO}_2\text{H}$  to  $\text{CONH}_2$ ), caerin 2.1 (Table 1) and caerin 1.10 for four male and four female *L. splendida*; or splendipherin and caerin 2.1 for four male and four female *Litoria caerulea*.

## RESULTS

#### HPLC

The peptide profiles of male and female *L. splendida* were monitored by HPLC and ESMS monthly over the three year period 1996–98. Similar results were obtained for each year. Examples of the HPLC traces are shown for male and female frogs for January 1998 (Fig. 1) and July 1998 (Fig. 2). The peptide components of the HPLC traces are designated 1–12 and their structures are listed in Table 1. The majority of these compounds have been identified previously [9]. Four components, however, have not been isolated previously from *L. splendida*. These have been identified and named as follows: Phe8 caerulein (compound 1), pEQDY(SO<sub>3</sub>)TGWDFD-NH<sub>2</sub>; desulfated caerulein (compound 2), pEQDYTGWMDF-NH<sub>2</sub>; splendipherin (compound 9), GLVSSIGKALGGLLADVVKSKGQPA-OH; and caerin 1.10 (compound 12), GLLSVLGSV-AKHVLPHVVPVIAEKL-NH<sub>2</sub>.

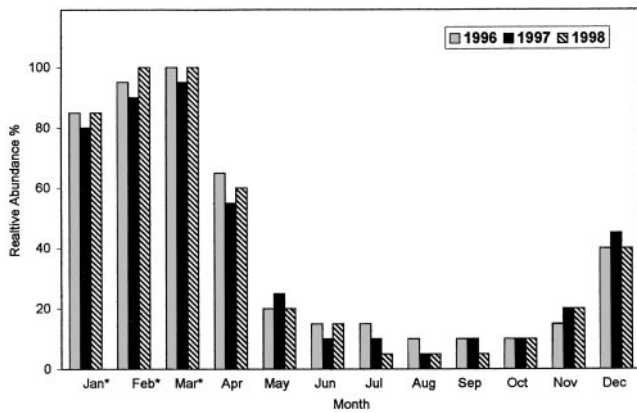


Fig. 4. Seasonal variation of splendipherin concentration for the years 1996–98. \*Denotes the breeding period of *L. splendida*.

Two of these compounds, splendipherin and caerin 1.10, are found only in the secretion of the male. The concentration of caerin 1.10 in the male secretion is constant during the year. In contrast, the concentration of splendipherin varies seasonally: results are summarized in Fig. 4. The composition of the neuropeptide (caerulein) region shown in Figs 1 and 2, although identical for both male and female, also varies seasonally. The seasonal variations of caerulein and Phe8 caerulein are shown in Fig. 5. The decrease in caerulein in the May to July period is accompanied by a corresponding increase in desulfated caerulein in the same period.

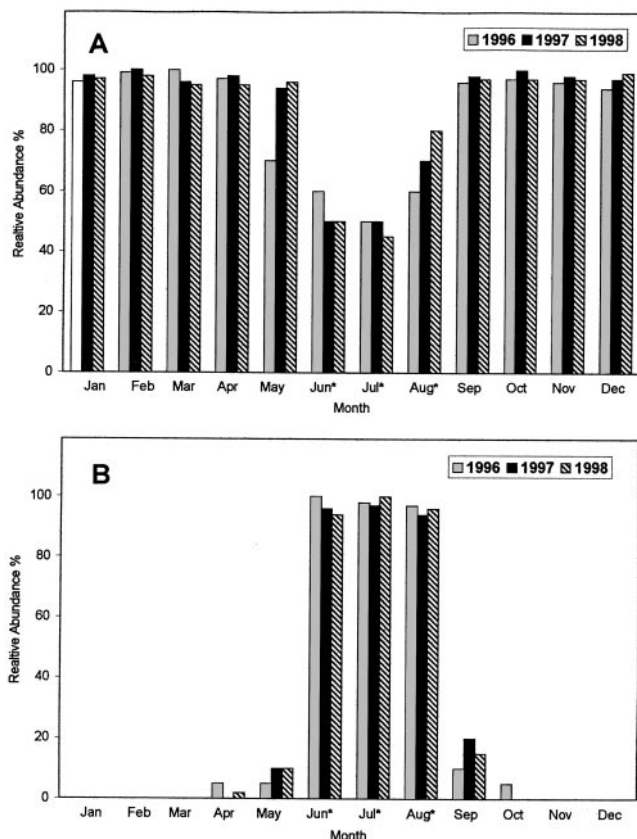


Fig. 5. Seasonal variation of (A) caerulein and (B) Phe8 caerulein relative concentrations for the years 1996–98. \*, Denotes winter months.

## Structure determination of peptides

The eight peptides listed in Table 1 have been isolated from *L. splendida* previously [6–9], and desulfated caerulein is also a known compound [2]. Splendipherin is identical with caerin 2.3, a major component of the secretion of *L. caerulea* [21]. These 10 peptides were shown to be identical in structure to the known compounds by comparison of HPLC and ESMS data. The new compounds were identified as outlined below.

**Phe8 caerulein.** This compound was identified by ESMS. It exhibits  $MH^+$  and  $(MH^+ - SO_3)$  ions at  $m/z$  1368 and 1288, respectively. Mass values used here are nominal values minus the sum of the integral masses of all the constituent amino acid residues. The mass spectrum (MS/MS) of the  $(MH^+ - SO_3)$  ion of Phe8 caerulein is shown in Fig. 6; the B and Y + 2 fragment ions identify the amino acid sequence (see [22] for details of fragmentations of peptides). This sequence cannot be confirmed by automated Edman sequencing because of the presence of the N-terminal pyroglutamate residue. To date we have been unable to obtain synthetic Phe8 caerulein because removing the peptide from the resin in the final step of the synthesis hydrolyses the sulfate of the tyrosine sulfate residue. Desulfated Phe8 caerulein (pEQDYTGWMDF-NH<sub>2</sub>) has been synthesized however, and the HPLC and ESMS data for the synthetic compound are identical with those of the desulfated peptide produced by mild acid hydrolysis of natural Phe8 caerulein.

**Caerin 1.10.** ESMS of caerin 1.10 gives an  $MH^+$  at 2573 Da and an  $[M + 2H]^{2+}$  ion at 1287 Da. The MS/MS data for the  $[M + 2H]^{2+}$  ion of caerin 1.10 are listed in Table 2. This provides the complete sequence except for the arrangement of the first four and the last two residues. A Lys-C digest gives two peptides,  $MH^+$  1043 and 1549 Da, whose MS/MS data (Table 2), together with those derived from the  $[M + 2H]^{2+}$  spectrum provide the full sequence of caerin 1.10, except that isomeric leucine and isoleucine cannot be differentiated by this technique. These residues were identified by automated Edman sequencing, which also confirms the total sequence of the peptide. Caerin 1.10 was synthesized, and natural and synthetic caerin 1.10 were shown to be identical by HPLC and ESMS comparison.

## Synthesis of peptides

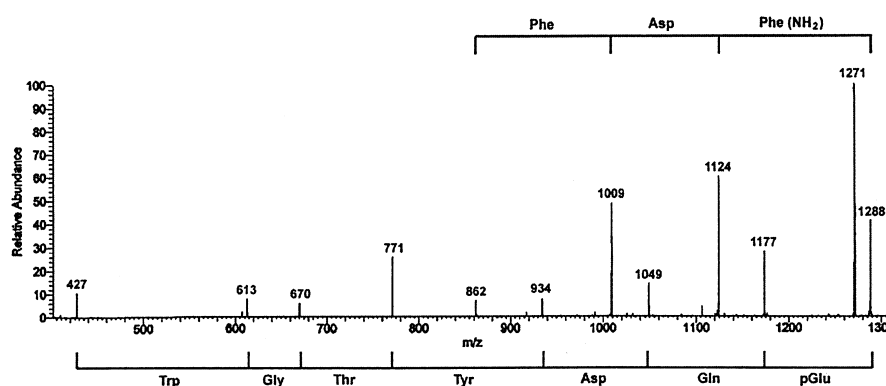
The peptides splendipherin, the derivative of splendipherin with the C-terminal CO<sub>2</sub>H changed to CONH<sub>2</sub>, caerin 2.1, caerin 1.10, desulfated caerulein and desulfated Phe8 caerulein, were synthesized from glycine and L-amino acids by Chiron Mimotopes (Clayton, Victoria) by standard techniques [20] in order to compare them with the natural peptides, and/or to provide sufficient material for antibiotic and behavioural testing.

## Antibiotic testing

Antibiotic testing was carried out using splendipherin, caerin 2.1 and caerin 1.10 by the microbiology section of the Institute of Medical and Veterinary Science (Adelaide). These data are recorded in Table 3 and are compared with data obtained previously [5] for the other major skin peptides of *L. splendida*, i.e. caerin 1.1 and 1.6, caerin 2.1 and caerin 3.1. Caerin 1.10 is a wide-spectrum antibiotic with activities slightly lower than those of caerin 1.1 and 1.6. In contrast, splendipherin shows no

Fig. 6. Electrospray mass spectrum (MS/MS) of the  $[MH^+ - SO_3]$  ion of Phe8 caerulein.

Schematics above the spectrum indicate the amino acid sequence derived from B fragmentations, while the data below the spectrum are derived from Y + 2 fragmentations. For a description of the mass spectral fragmentations of peptides, see [22].



antibiotic activity (inactive in this context is defined as  $MIC > 100 \mu\text{g}\cdot\text{mL}^{-1}$  against all the test organisms).

### Behavioural testing

**Splendipherin.** The female *L. splendida* was allowed to sit for five minutes in the centre of a two metre aquarium containing water to a depth of 2 cm. Addition of 40 ng (a tank concentration of  $10^{-11}$  M) of splendipherin in water to a gauze pad situated at one end of the tank and one metre from the frog elicits a distinct change in posture and an increased degree of alertness in the animal within 20 s of introduction of the pheromone. The female then walks slowly towards the pad, sits on it, and remains seated until removed. The animal does not walk in a straight line towards the pad containing the pheromone, she moves in an arc either to the left or right of her initial position. At the midpoint of the arc she is within 5–10 cm of the side of the tank; she then completes the arc approaching the swab either from the right or the left as appropriate. The pathways of individual animals are not precisely the same but do follow the same general trend. This

experiment has been done separately with four different females with the pheromone added to either of the two swabs shown in Fig. 3A. The success rate is 100% and the average time from introduction of the pheromone until the time the female sits on the gauze pad is 7 min. An example of one such test is shown in pictorial form in Fig. 3. When the amount of pheromone added to the pad is greater than 4000 ng, the female becomes confused and unable to find the source of the pheromone. Decreasing the amount of pheromone to below 4 ng elicits no response from the female.

Splendipherin effects no behavioural response, not even minor aggressive behaviour, in male *L. splendida* using identical behavioural tests.

**Other peptides.** Neither caerin 2.1 (Table 1), which is very similar in structure to splendipherin (Arg8 replaces Lys8), nor the synthetically modified splendipherin (C-terminal  $\text{CO}_2\text{H}$  replaced by  $\text{CONH}_2$ ) produce any response from either male or female *L. splendida* in behavioural tests identical with those outlined above. Caerin 2.3 (now renamed splendipherin) is a major peptide constituent of the glandular secretion of the

**Table 2.** The structure determination of caerin 1.10.  $MH^+ = 2573$ . Esterification [7] gives a methyl ester,  $MH^+ = 2606$ , therefore caerin 1.10 contains one  $\text{CO}_2\text{H}$  and one  $\text{CONH}_2$  group.

Procedure (in order)	Ions	<i>m/z</i>	Sequence	Overall sequence for each stage
[M + 2H <sup>+</sup> ] <sup>2+</sup> ( <i>m/z</i> 1288)				
MS/MS	B	2315, 2186, 2115, 2002, 1903, 1806, 1707, 1608, 1471, 1374, 1261, 1162, 1025, 897, 826, 727, 640, 583	[GSVAKHVLPHVVPVIAE]	
	Y + 2	2203, 2104, 1991, 1934, 1847, 1748, 1677, 1549, 1412, 1313, 1200, 1103, 966, 867, 768, 671	[VLGSVAKHVLPHVVP]	
Lys-C digestion gives $MH^+$ 1043, 1437 and 1549				
$MH^+ = 1043$				
MS/MS	B	1021, 897, 826, 727, 640, 583, 470	[LGSVAK]	
	Y + 2	1043, 986, 873, 760, 673, 574, 461	[GLLSVL]	GLLSVLGSVAK
$MH^+ = 1549$				
MS/MS	B	1532, 1419, 1291, 1162, 1091, 978, 879, 782, 683, 584, 447	[HVVPVIAEKL-NH <sub>2</sub> ]	
	Y + 2	1549, 1412, 1313, 1200, 1103, 966, 867, 768, 671, 572	[HVLPHVVPV]	HVLPHVVPVIAEKL-NH <sub>2</sub>
Complete caerin 1.10				GLLSVLGSVAKHVLPHVVPVIAEKL-NH <sub>2</sub>

**Table 3. Antibiotic activity of peptides from *L. splendida*.** MIC values in  $\mu\text{g}\cdot\text{mL}^{-1}$ ; if no value is indicated then MIC  $> 100 \mu\text{g}\cdot\text{mL}^{-1}$ . The peptides are: caerin 1.1 (C1.1), GLLSVLGSVAKHVLPHVVPVIAEHL-NH<sub>2</sub>; caerin 1.6 (C1.6), GLFSVLGAVAKHVLPHVVPVIAEKL-NH<sub>2</sub>; caerin 1.10 (C1.10), GLLSVLGSVAKHVLPHVVPVIAEKL-NH<sub>2</sub>; caerin 2.1 (C2.1), GLVSSI-GRALGGLLADVVKSKGQPA-OH; splendipherin (Spl.), GLVSSIGKAL-GLLADVVKSKGQPA-OH; caerin 3.1 (C3.1), GLWQKIKDKASELVS GIVEGVK-NH<sub>2</sub>.

Organism	C1.1	C1.6	C1.10	C2.1	Spl.	C3.1
<i>Bacillus cereus</i>	50					
<i>Escherichia coli</i>		50				
<i>Leuconostoc lactis</i>	1.5	3	6			
<i>Listeria innocua</i>	25	50	50			
<i>Micrococcus luteus</i>	12	25	25			< 0.4
<i>Pasteurella multocida</i>	25	25	100	25		
<i>Staphylococcus aureus</i>	3	6				
<i>Staphylococcus epidermidis</i>	12	12	100			
<i>Streptococcus uberis</i>	12	25	50			

related Australian Green Tree Frog *Litoria caerulea* [21], but it does not induce any behavioural response in similar aquatic behavioural tests with either the female or male of this species.

Finally, the wide-spectrum antibiotic caerin 1.10 produces no effect with either male or female *L. splendida* in aquatic behavioural tests.

## DISCUSSION

### The antibiotic caerin 1.10

The male *L. splendida* produces caerin 1.10, a minor component that is not present in the glandular secretion of the female. This peptide is a wide-spectrum antibiotic showing activity similar to, but not as strong as, those of the two major antibiotic components, caerin 1.1 and caerin 1.6 (which are present in the secretions of both male and female) [5,23]. Caerin 1.10 shows no pheromone activity, so the reason for its presence specifically in the male secretion is not clear. The concentration of this peptide is constant throughout the year.

### Seasonal changes in neuropeptides

The seasonal variations of the excreted neuropeptides of *L. splendida* (Fig. 5) are remarkably consistent over the three-year test period, and are identical for the male and female. Caerulein is the sole neuropeptide formed in the period September to May. This is the (southern hemisphere) spring to autumn period and includes the breeding period of *L. splendida* (normally January to March). Several striking changes occur in the winter period of June to August. The caerulein concentration diminishes in the secretion and this drop is counterbalanced by the formation of desulfated caerulein. The decrease in caerulein formation is also precisely matched by the production of a new peptide, Phe8 caerulein. Interestingly, there is no desulfated Phe8 caerulein co-occurring with Phe8 caerulein. The precise reasons for these seasonal changes are not known. Caerulein is a potent vasodilator, analgaesic and hormone [1–3], and it seems likely that Phe8 caerulein should show corresponding activity. We have been hampered in this investigation because we have been unsuccessful in obtaining synthesized Phe8 caerulein in order to have sufficient material

to enable activity tests to be performed. Even though there is no detectable natural desulfated Phe8 caerulein in the glandular exudate, synthetic Phe8 caerulein is readily hydrolysed when the synthetic peptide is removed from the resin support in the last step of the synthesis. There could be two reasons why caerulein is being hydrolysed by a specific endoprotease that does not similarly hydrolyse Phe8 caerulein: to reduce the amount and consequently the overall activity of caerulein; or to form desulfated caerulein, which has some specific role in the amphibian integument. The former reason seems unlikely for two interrelated reasons. First, if deactivation of caerulein is required, surely the endoprotease would remove all of the caerulein. Second, even when the concentration of caerulein is at its lowest (June to August), there is still some 5 mg of this material in the glandular exudate, and caerulein is active against smooth muscle at concentrations  $\leq 10^{-9}$  M. Desulfated caerulein shows no smooth muscle activity [1], so any specific role it may have can have nothing to do with vasodilator activity. Desulfated caerulein and desulfated Phe8 caerulein have been synthesized and tested for a range of bioactivities. These two compounds show no antibiotic, anticancer, N-type Ca<sup>2+</sup> channel, L-type Ca<sup>2+</sup> channel, neuronal NOS, serotonin receptor, DNA methyl green, or nitric oxide scavenging activity.

The neuropeptide changes occur during the winter months when *L. splendida* is inactive in captivity or hibernating in the natural habitat. It is known that neuropeptides are involved in thermoregulation in animals [24–29], including toads [29], so the probability is that these complex seasonal changes in neuropeptide content are somehow involved in the thermogenesis of *L. splendida*.

### The male sex pheromone splendipherin

Splendipherin attracts female *L. splendida* in water at a (tank) concentration range of  $10^{-11}$ – $10^{-9}$  M. The frog cannot detect the pheromone at concentrations below  $10^{-13}$  M, and if the concentration of pheromone is  $\geq 10^{-8}$  M, she becomes confused and cannot find the source of the pheromone. Natural splendipherin and synthetic L-splendipherin show identical pheromone activity. We have investigated several peptides structurally similar to splendipherin in similar aquatic behavioural tests and they show no pheromone reactivity. Caerin 2.1, which differs from splendipherin only at residue 8 (arginine instead of lysine), and peptide GLVSSIGKALGGLLADVVKSKGQPA-NH<sub>2</sub>, which differs from splendipherin only in that C-terminal CONH<sub>2</sub> replaces CO<sub>2</sub>H, show no pheromone activity. As splendipherin has no behavioural affect on the related species *L. caerulea*, we may conclude that splendipherin is the species-specific sex pheromone of male *L. splendida*.

The female recognizes the presence of the pheromone some 20 s after it is introduced into the swab one metre away from the animal. She does not hurry towards the pheromone source, but moves sedately through the shallow water in an angular approach towards the pheromone as shown in Fig. 3. We have also examined the behaviour of two females moving towards the same pheromone source; they appear not to compete, but both move forward at normal speed. The speed of recognition of the female for the pheromone cannot be a consequence of the pheromone diffusing through one metre of still water, because such diffusion is slow. The speed of pheromone recognition, together with the unusual angular approach of the female to the pheromone source in the tank, suggests that the pheromone may be moving as a surfactant on the surface of the water with some

build up in surface concentration of the pheromone towards the walls of the tank.

## CONCLUSIONS

In terms of the stated aims of this project, we have shown that the skin peptide profile of *L. splendida* varies seasonally and shows marginal differences for male and female. Care must therefore be taken when using the skin peptide profile of an anuran as a probe to attempt to identify closely related species or different populations of a particular species. If such a technique is to be used, the sex of the frog providing the secretion must be known, and the peptide profile under investigation and the measured standard (of a frog of known gender) must be of secretions taken at the same time of the year. We have also identified the peptide named splendipherin as the aquatic male sex pheromone of *L. splendida*. As the aquatic newt *C. pyrrhogaster* also has a peptide as an aquatic male pheromone [16], it is likely that other amphibians that breed in water may also have their own species-specific aquatic sex pheromones.

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