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Intersexual variations in Northern (*Missulena pruinosa*) and Eastern (*M. bradleyi*) mouse spider venom ☆

Volker Herzig[a,*], Ali A. Khalife[b], Youmie Chong[b], Geoffrey K. Isbister[c], Bart J. Currie[c], Tracey B. Churchill[d], Suzanne Horner[d], Pierre Escoubas[e], Graham M. Nicholson[b], Wayne C. Hodgson[a]

[a]*Monash Venom Group, Department of Pharmacology, Monash University, Victoria, Australia*
[b]*Neurotoxin Research Group, Department of Medical & Molecular Biosciences, University of Technology, Sydney, NSW, Australia*
[c]*Tropical Toxinology Unit, Menzies School of Health Research, Darwin, Northern Territory, Australia*
[d]*Museum and Art Gallery of the Northern Territory, Darwin, Northern Territory, Australia*
[e]*University of Nice, Sophia Antipolis, Institut de Pharmacologie Moléculaire et Cellulaire CNRS, Valbonne, France*

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Abstract

Venoms of both sexes of Australian Northern (*Missulena pruinosa*) and Eastern (*Missulena bradleyi*) mouse spiders were studied in order to determine intersexual variations in venom yield, composition and bioactivity. Females of both species yielded more venom than males. High-performance liquid chromatography (HPLC) and mass spectrometry data further indicate a substantial degree of intersexual variation in the venom composition of both species. In a cricket (*Acheta domestica*) acute toxicity assay, only small intersexual differences were observed, but *M. bradleyi* venom was found to be considerably more potent than *M. pruinosa* venom. In the chick biventer cervicis nerve–muscle preparation, male but not female *M. bradleyi* venom induced large and sustained muscle contractions with fasciculation and decreased twitch height that could be reversed by CSL funnel-web spider antivenom. In contrast, venoms of both sexes of *M. pruinosa* did not induce significant effects in the chick biventer cervicis nerve–muscle preparation. We therefore conclude that female *M. bradleyi* venom and venoms from male and female *M. pruinosa* appear to contain few, if any, orthologs of δ-missulenatoxin-Mb1a, the toxin responsible for the effects of male *M. bradleyi* venom in vertebrates. These findings are consistent with clinical reports that mouse spiders, particularly species other than male *M. bradleyi*, do not appear to be a major medical problem in humans.

Keywords: *Missulena pruinosa*; *Missulena bradleyi*; Mouse spider; Actinopodidae; Venom; Intersexual variations

1. Introduction

Mouse spiders of the genus *Missulena* (Araneae, Actinopodidae) are an ancient group of mygalomorph spiders with 11 known species (10 from Australia and one from Chile) (Platnick, 2007).

☆ *Ethical statement*: The authors declare that all animal experiments described in the paper comply with Australian animal ethics regulations.

*Corresponding author. Tel.: +61 3 9905 4866; fax: +61 3 9905 5851.
E-mail address: schnakenstich@yahoo.com (V. Herzig).

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Indeed, they represent one of the earliest forms of spiders in Australia (Main and Main, 1956). According to Brunet (1997), the name 'mouse spider' refers to the behaviour of the female to dig burrows that reach more than 1 m deep. Bites from the Eastern mouse spider (*Missulena bradleyi*) are known to be clinically significant and potentially result in severe envenoming resembling that of Australian funnel-web spiders (Rendle-Short, 1985; Isbister, 2004). The molecular determinants of toxicity in *M. bradleyi* are not well known; however, one toxin (δ-missulenatoxin-Mb1a) has been identified and was shown to be 88% homologous with the lethal neurotoxin δ-atracotoxin-Ar1a from the Sydney funnel-web spider *Atrax robustus* (Gunning et al., 2003).

Another species from the genus *Missulena* is the Northern mouse spider (*M. pruinosa*) that was originally described by Levitt-Gregg (1966) and occurs in the Northern parts of Australia (for a distribution map see Nicholson et al., 2006). Female *M. pruinosa* are completely black with a shiny prosoma. Adult males are also black with a shiny prosoma, but the dorsal surface of their opisthosoma is characteristically white coloured. Due to the cryptic life of *M. pruinosa*, encounters and therefore biting accidents with this species are quite rare with approximately four biting accidents in the Darwin region per year (Isbister et al., 2001). During the build-up of the wet season (mainly during September and October) adult male *M. pruinosa* leave their burrow to seek females with whom to mate. This is consistent with the seasonally restricted pattern of biting accidents as reported by Isbister (2004).

No pharmacological and biochemical studies have so far been carried out on *M. pruinosa* venom. According to clinical reports, the most frequent symptoms of *M. pruinosa* bites are minor local effects, but in some cases systemic effects such as headaches and nausea can be observed (Isbister et al., 2001; Isbister and Gray, 2004). Generally, severe envenoming from mouse spiders is rare (estimated to occur in 2.5% of all cases according to Isbister, 2004) and the overall number of biting accidents involving *M. pruinosa* is rather low. Due to its close taxonomic relationship with *M. bradleyi*, it might be assumed that *M. pruinosa* venom contains toxins that may be potentially dangerous to vertebrates (including humans). Therefore, we have studied the biochemical and pharmacological properties of the venoms from *M. pruinosa* and *M. bradleyi*, with emphasis on intersexual variations. Venom profiles were determined by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Invertebrate venom activity was tested in crickets while the isolated, electrically-stimulated chick biventer cervicis nerve–muscle preparation served as a bioassay to investigate venom effects on vertebrate neuromuscular activity.

2. Materials and methods

2.1. Spider collection and venom preparation

Two female and 101 male *M. pruinosa* were collected in the Darwin region (Northern Territory, Australia) during the build-up of the wet season in October 2005. Venom was extracted by applying electrostimulation (9–12 V, 3–5 s per side) to the basal parts of the chelicerae. The expressed venom was collected from the tips of the fangs and immediately stored frozen at −20 °C. All venoms were then freeze-dried and stored at −75 °C for further use. The venoms were pooled according to the spider's sex, centrifuged at 14,000 rpm for 4 min and the supernatant was filtered through an ion-disc filter (N-13-4, nylon, 13 mm × 0.45 μm, Supelco, Supelco Park, Bellefonte, PA, USA) before being analysed.

Male and female *M. bradleyi* were collected from suburban areas in the Central Coast region and Newcastle, NSW, Australia. Male *M. bradleyi* spiders were aggravated and venom was then aspirated directly from the chelicerae into a 100 μL micropipette tip and then transferred to an Eppendorf tube containing distilled water. The venom was then stored at −20 °C until required. The female spiders were initially electrostimulated in order to aspirate the venom, as only 15% voluntarily expressed any venom at their fang tips after aggravation. Venom was extracted by applying electrostimulation to the basal parts of the chelicerae. A square wave electrical pulse (10–30 V, 2 ms, 40 Hz) was applied across the chelicerae, using a Digitimer Stimulator DS9A, with the aid of electrode gel (TGS Sonic-Gel) in order to increase electrical conductivity. The venom collection method for female *M. bradleyi* was eventually altered to increase venom yield. Spiders were forced to bite around a section of polyethylene plastic tubing and venom was collected using a micropipette from the surface of the tubing and fangs. Another advantage of this procedure was that it prevented contamination

of the venom sample with gut contents and saliva, which may be regurgitated during electrostimulation. The venom was then stored at −20 °C until required.

2.2. Spider identification

Expert identification of *M. pruinosa* spiders was carried out by Tracey B. Churchill at the Museum and Art Gallery of the Northern Territory, Darwin, Australia. *M. bradleyi* were identified by Mike Gray at the Australian Museum in Sydney.

2.3. Reversed-phase high-performance liquid chromatography

The fractionation of *M. pruinosa* and *M. bradleyi* venom was carried out using reversed-phase HPLC (rp-HPLC). *M. pruinosa* venom was separated using a Phenomenex Jupiter analytical C_{18} rp-HPLC column (15 cm × 2 mm, 5 μm, Phenomenex Australia Pty. Ltd., Sydney, NSW, Australia) connected to a Shimadzu 10A series HPLC system. Peptide elution was monitored at 214 nm. The flow rate was 0.2 mL/min and varying linear gradients of eluent A (0.1% trifluoracetic acid) and eluent B (90% acetonitrile + 0.09% trifluoracetic acid) were used. The percentage of eluent B was increased from 0% to 5% in 5 min, followed by an increase from 5% to 65% in 55 min. *M. bradleyi* venom was separated using a Vydac analytical C_{18} rp-HPLC column (25 cm × 4.6 mm, 5 μm) at a flow rate of 1 mL/min and the absorbance was monitored at 215 nm. A linear gradient of 5–60% acetonitrile + 0.1% trifluoracetic acid over 55 min was used. Peak areas were analysed using the Shimadzu Class-VP 6.12 SP3 software (for *M. pruinosa*) or Prism 4.0 (GraphPad Software Inc.; for *M. bradleyi*). Only peaks comprising at least 0.2% of the total area of all peaks were used to determine intersexual variations in the venom composition of each species.

2.4. Mass spectrometry

MALDI-TOF mass spectrometry was performed on a Voyager DE-Pro instrument (Applied Biosystems, Foster City, USA) operated in linear or reflector mode. Crude venom samples were dissolved in 10 μL HPLC-grade water and 0.5 μL were mixed on-plate in a 1:2 ratio with α-cyano-4-hydroxycinnamic acid matrix made up in acetonitrile/water/trifluoroacetic acid (50:50:0.1) (5 mg/mL). Spectra were externally calibrated with a mixture of synthetic peptides (1000–4000 Da mass range) using the same matrix system. Spectra were processed in the Data Explorer software (Applied Biosystems, Foster City, USA) and where appropriate, masses of known toxins were calculated with the GPMAW software (Lighthouse Software, Odense, Denmark).

2.5. Cricket toxicity assay

We used a method that was similar to that applied by Chong et al. (2007), but with some modifications. The paralysing and lethal activities of *M. pruinosa* and *M. bradleyi* venoms were assessed by thoracic injection of the venom into 3rd–4th instar house crickets (*Acheta domestica*). Crickets ($n = 10$ for each venom dose and the respective control for *M. bradleyi* and $n = 5$ for *M. pruinosa*) of undetermined sex (Pisces Enterprises Pty. Ltd., Brisbane, Qld., Australia) weighing 60–100 mg were used. Three tests were carried out for female *M. bradleyi* venom and only one test for male *M. bradleyi* and male and female *M. pruinosa* venom. Venom was injected into the ventro-lateral thoracic region between legs 2 and 3 using a 1 mL Terumo Insulin syringe (B-D Ultra-Fine, Terumo Medical Corporation, MD, USA) with a fixed 29 G needle fitted to an Arnold hand microapplicator (Burkard Manufacturing Co. Ltd., England). All venoms were dissolved in insect saline (Eitan et al., 1990) of the following composition: (in mM): 200 NaCl, 3.1 KCl, 5.4 $CaCl_2$, 4 $MgCl_2$, 2 $NaHCO_3$, 0.1 Na_2HPO_4, with the pH adjusted to 7.2 using NaOH, and supplemented with 0.1% bovine serum albumin (fraction V) to reduce non-specific peptide interaction with glassware. Controls received the maximum injection volume, i.e. 5 μL of insect saline per cricket. After the injection, crickets were placed in small plastic containers with food and water, and monitored at regular time intervals (0.5, 1, 2, 3, 4, 5, 6, 24, 48 and 72 h post-injection) for signs of paralysis or death. Crickets showing no severe movement or posture dysfunction were categorized as 'normal'. Two additional categories were used: 'knockdown' (i.e. immobile, inability to maintain normal posture, intermittent or continuous twitches of appendages) and 'lethal' (i.e. no signs of movement following mechanical stimulation of the appendages and the body).

For determination of KD_{50} (median knockdown dose) and LD_{50} (median lethal dose), time points of 24, 48 and 72 h after venom injection were chosen and the control crickets affected at that time point were subtracted from the number of crickets affected in the venom-treated groups. The corrected numbers were then up-scaled to a 100% scale by multiplying all percentages with a control factor (defined as 100/(100−percentage of control crickets affected at the respective post-injection time)). The resulting percentages were used to interpolate the KD_{50} and LD_{50} values by fitting log dose–response curves using non-linear regression analysis in Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA) according to the following logistic function:

$$Y = \frac{100}{1 + 10^{(\log \text{Dose}_{50} - X)n_H}} \quad (1)$$

where Y is the percentage affected (either knockdown or lethal) at a given time point, X is the logarithm of the venom dose (in μg of venom per gram of cricket), Dose_{50} is either the KD_{50} or LD_{50}, and n_H represents the Hill coefficient (the variable slope factor).

2.6. Isolated chick biventer cervicis nerve-muscle preparation

Biventer cervicis muscles were removed from male chicks (2–15 days-old), mounted in 5-mL organ baths and maintained at 34 °C under 1 g resting tension in a physiological saline solution of the following composition (in mM): 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, 11.1 D-glucose and bubbled continuously with 95% O_2/5% CO_2. Isometric contractions were measured via a Grass transducer (FTO3) connected to a Powerlab 4/20 or MacLab/4s system (ADInstruments, NSW, Australia). Twitches were evoked by stimulating the motor nerve (supramaximal voltage, 0.05–0.2 ms, 0.1 Hz) via silver electrodes connected to a Grass S88 stimulator. Nerve-mediated (indirectly-evoked) twitches were confirmed by the addition of *d*-tubocurarine (10 μM). In the absence of electrical stimulation, responses to exogenous acetylcholine (1 mM, 30 s), carbachol (0.02 mM, 60 s) and KCl (40 mM, 30 s) were obtained prior to the addition of venom and at the conclusion of the experiment. Preparations were allowed to equilibrate for at least 20 min with continuous stimulation before the addition of venom. Venoms were diluted in Milli-Q water and the maximum volume added to the bath was 50 μL.

2.7. Drugs and chemicals

Agar, acetylcholine chloride, carbamylcholine chloride (carbachol) and *d*-tubocurarine chloride were obtained from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of drugs were made up in Milli-Q water unless otherwise stated. Funnel-web spider antivenom was purchased from CSL Ltd. (Melbourne, Australia).

2.8. Statistics

For all statistical analyses the program SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA) was used and significance levels were set to 0.05. For the chick biventer study, a two-way analysis of variance based on repeated measures using the factors venom concentration and time followed by Holm–Sidak post-hoc comparisons was applied to determine venom effects on baseline and twitch height. *T*-tests were used to compare the muscle responses to exogenous acetylcholine, carbachol and KCl before and after addition of the venom. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Spiders and venom yield

The two adult female *M. pruinosa* in this study showed a body length (i.e. from the distal tip of the basal part of the chelicerae to the tip of the abdomen) of 23–26 mm and a legspan (i.e. from the distal tip of leg 1 on one side to the distal tip of leg 4 on the other side with legs stretched out) of 45–47 mm. The adult males of *M. pruinosa* showed a larger variation in their size with a body length of 13–20 mm and a legspan of 40–53 mm. The total yield of dried venom was 680 μg of female venom (from three milkings, i.e. 227 μg/spider) and 1.64 mg of male venom (from 230 milkings. i.e. 7 μg/spider). For *M. bradleyi* it was calculated that male specimens produced an average of 10 μg of venom protein per milking, while female specimens produced an average of 65 μg.

3.2. HPLC

Analysis of the HPLC chromatograms of *M. pruinosa* venom (Fig. 1A) revealed a total of 35 different peaks (>0.2% of total peak area), with 23

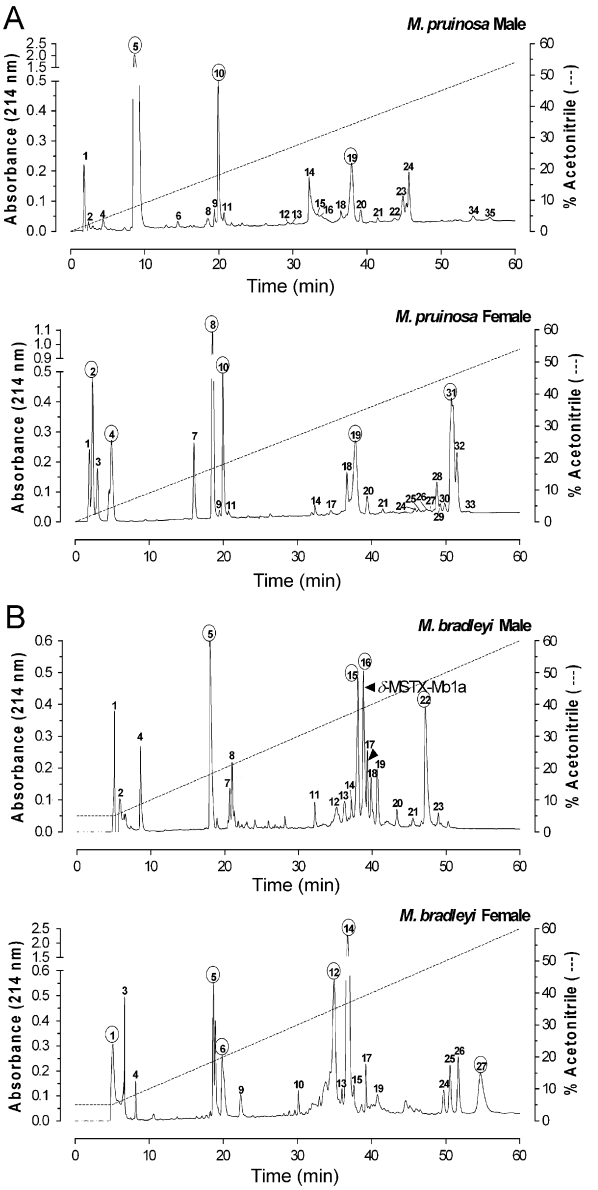

Fig. 1. rp-HPLC chromatograms of pooled venoms from male and female (A) *M. pruinosa* and (B) *M. bradleyi* indicating absorbance (solid line) and the percentage of acetonitrile (dashed line) over a time period of 60 min. Numbers indicate peaks with >0.2% of the peak area (of all peaks) and encircled numbers denote major peaks (>5% of the peak area). All peak numbers are based on the comparison of the retention times within each species; so they are not applicable for between-species comparison. Grey arrowheads indicate crude fractions with insecticidal activity while black arrowheads indicate vertebrate active toxins including δ-missulenatoxin-Mb1a (δ-MSTX-Mb1a) in male *M. bradleyi* venom.

peaks being present in male and 25 peaks in female venom. The number of major peaks (>5% of total peak area) was 3 in male and 6 in female *M. pruinosa* venom, with two peaks (peak 10 and 19) being major peaks in the venoms of both sexes. The most striking intersexual differences were observed in peak 5 (peak area of 64.3% (male) vs. <0.2% (female)), peak 8 (0.7% vs. 20.6%) and peak 31 (<0.2% vs. 15.7%).

A total of 27 different peaks were found in the *M. bradleyi* venoms (Fig. 1B), with 19 peaks being present in male and 17 peaks in female venom. The number of major peaks was 4 in male and 6 in female *M. pruinosa* venom, with one peak (peak 5) being a major peak in the venoms of both sexes. The biggest quantitative intersexual differences between male and female spider venom were found in peak 5 (23.4% vs. 10.2%), peak 12 (0.5% vs. 10.7%), peak 14 (0.4% vs. 51.9%), peak 15 (16.6% vs. 0.9%), peak 16 (18.4% vs. <0.2%) and peak 22 (15.2% vs. <0.2%).

3.3. Mass spectrometry

Observation of MALDI-TOF mass spectra (Fig. 2) shows two distinct results. The venoms of male and female *M. pruinosa* (Fig. 2A) appear almost identical, dominated by a high-intensity ion at ca. *m*/*z* 4111. Considering slight measurement differences induced by external calibration, it seems that both venoms may possess the same major component although the molecular identity of these peptides would need to be established via Edman sequencing for confirmation. Other venom components fall in the 3000–8000 *m*/*z* range. The venoms of male and female *M. bradleyi* (Fig. 2B) appear to show more differences although all observed ions fall into the same *m*/*z* range. The main ion in male venom is observed at *m*/*z* 3708.9, while in female venom it is at *m*/*z* 4157.9. Neither δ- nor ω-missulenatoxin-Mb1a (Gunning et al., 2003; Chong et al., unpublished data) could be detected at respectively *m*/*z* 4934.8 and 4167.5 in the spectrum of crude *M. bradleyi* venom.

3.4. Effects of the venom in crickets

The results of the toxicity tests in crickets show that venom from both male (Fig. 3A) and female (Fig. 3B) *M. pruinosa* produced knockdown and lethality after 48 h. The respective KD_{50} values were quite similar between both sexes (47.3 μg/g for male, 53.2 μg/g for female venom), while a more pronounced difference was found for the LD_{50} values (male: 169.9 μg/g; female: 105.3 μg/g) at 48 h. In *M. bradleyi* venom (Fig. 3A–B), the difference in the KD_{50} values was even more pronounced

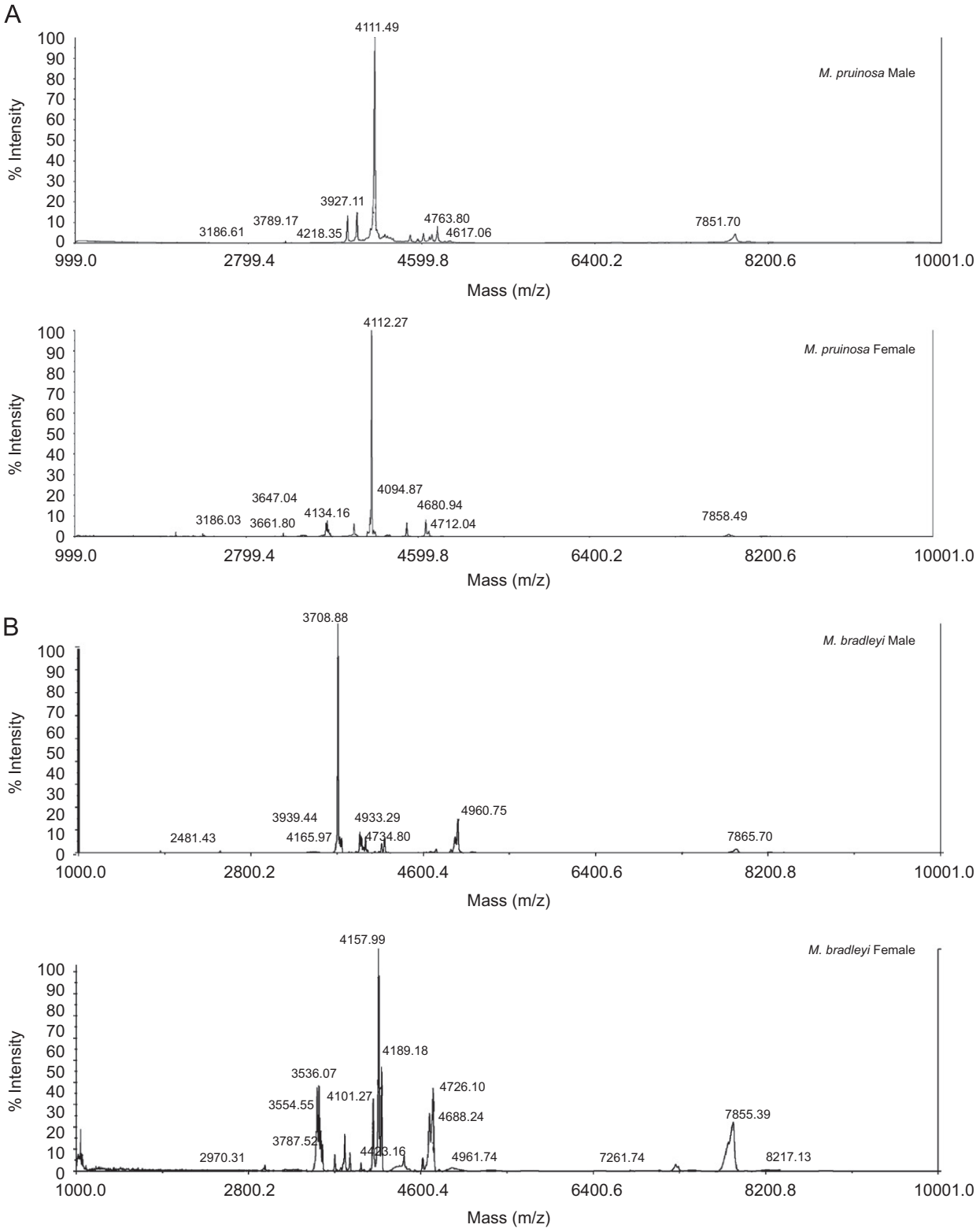

Fig. 2. MALDI-TOF mass spectra of pooled venoms from male and female (A) *M. pruinosa* and (B) *M. bradleyi*.

(male: 7.3 μg/g; female: 3.0 μg/g) compared to the respective LD_{50} values (male: 8.6 μg/g; female: 7.2 μg/g). Independent of species and sex, the difference between KD_{50} and LD_{50} values decreased with longer post-injection times within each species (Fig. 3C). The female *M. bradleyi* experiments in crickets were repeated three times and the data represent the mean ± SEM. Due to the small amounts of available venom, only one test was carried out per venom dose for male *M. bradleyi* and male and female *M. pruinosa*, excluding the use of proper statistics.

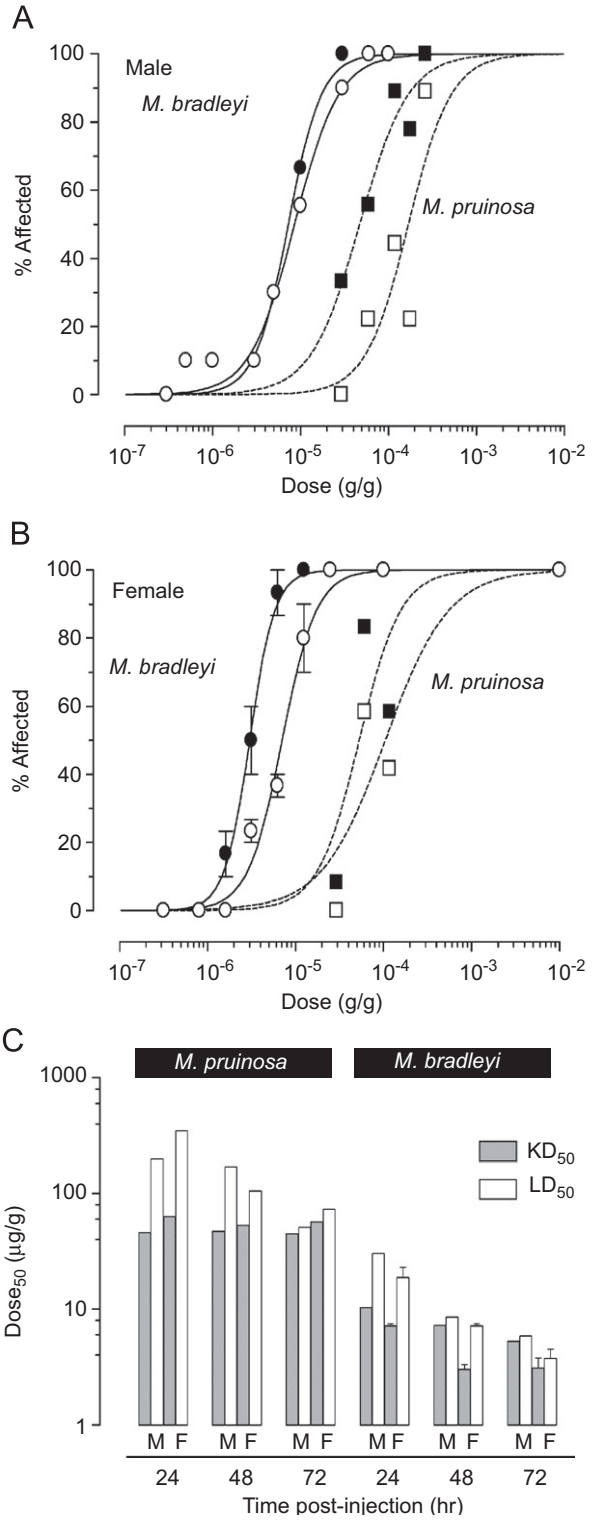

Fig. 3. Acute toxicity of *Missulena* venoms in house crickets (*A. domesticus*). (A–B) Log-dose response curve for knockdown (closed symbols) and death (open symbols) of crickets by *M. pruinosa* (squares) and *M. bradleyi* (circles) venom at 48 h post-injection. Panels represent data using (A) male and (B) female venoms, dose is indicated in grams of venom per gram of cricket. Data were fitted with Eq. (1) (see Section 2.5). (C) Effective dose (in μg of venom per gram of cricket) for 50% knockdown (KD_{50}, grey bars) or 50% death (LD_{50}, white bars) at 24, 48 or 72 h post-injection for male (M) and female (F) *M. pruinosa* and *M. bradleyi* venom. The female *M. bradleyi* experiments ($n = 10$ per venom dose) were repeated three times and the data represent the mean ± SEM. Due to the small amounts of available venom, only one test was carried out per venom dose for male *M. bradleyi* ($n = 10$) and male and female *M. pruinosa* ($n = 5$). Note the log scale in the *y*-axis in panel C.

3.5. Effects on the isolated chick biventer cervicis nerve–muscle preparation

The effects for *M. bradleyi* venom in the chick biventer cervicis assay have already been published in detail in Rash et al. (2000). Our results now confirm those previous results showing that low organ bath concentrations of male *M. bradleyi* venom (1.5 μg/mL) caused a small sustained contracture, as evidenced by an increase in baseline tension, followed by a small increase in twitch amplitude (Fig. 4A). Higher concentrations ranging from 3 μg/mL (Fig. 4B) to 6 μg/mL (Fig. 4C) produced marked muscle fasciculation, sustained contracture and a decrease in twitch amplitude. The addition of 1 unit/mL of funnel-web spider antivenom fully reversed the effects caused by the highest concentration of male *M. bradleyi* venom (Fig. 4C). Female *M. bradleyi* venom, at a concentration of 18 μg/mL, failed to alter twitch or baseline resting tension (Fig. 4D), which further underlines the results of Rash et al. (2000).

Statistical analysis of the effects of *M. pruinosa* venom on the chick nerve–muscle preparation indicates that the venom did not affect baseline muscle tension (Fig. 5A), revealing only significant effects for the factor time ($F_{9,234} = 69.4$; $P < 0.001$),

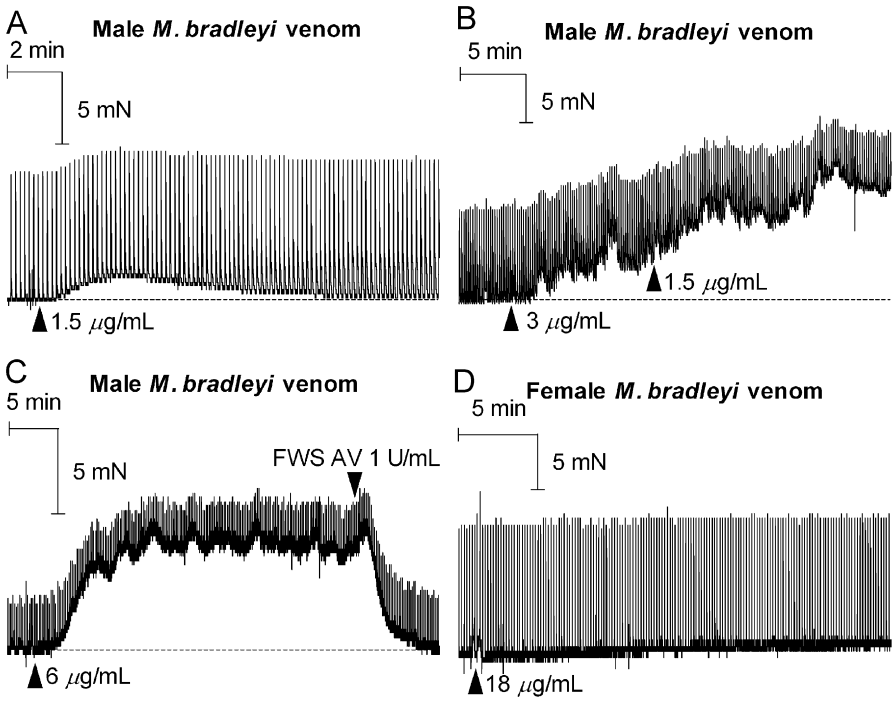

Fig. 4. Effects of *M. bradleyi* venom on the indirectly-stimulated chick biventer cervicis nerve–muscle preparation. (A–C) Representative traces showing dose-dependent effects of male venom. (A) At low concentrations, male venom (1.5 μg/mL) caused a small sustained contracture, as evidenced by an increase in baseline tension, followed by a small increase in twitch amplitude. (B–C) Higher concentrations, ranging from 3 μg/mL (B) to 6 μg/mL (C), produced marked muscle fasciculation, sustained contracture and a decrease in twitch amplitude. These effects could be fully reversed by the addition of 1 U/mL of Sydney funnel-web spider antivenom (FWS AV), as shown in panel C. (D) Female *M. bradleyi* venom, at a concentration of 18 μg/mL, failed to alter twitch or baseline resting tension.

but not for the venom concentration ($F_{6,234} = 1.97$; $P = 0.106$). Similar analysis of the twitch height data (Fig. 5B) revealed significant effects for *M. pruinosa* venom for the factors venom concentration ($F_{6,468} = 3.68$; $P = 0.009$), time ($F_{18,468} = 6.88$; $P < 0.001$) and the interaction of concentration × time ($F_{108,468} = 2.04$; $P < 0.001$). However, post-hoc comparisons for the factor concentration did not reveal any significant effects for the tested venom concentrations of both sexes. Additionally, no significant venom effects were detected in the non-stimulated response to exogenous acetylcholine, carbachol and KCl after the addition of *M. pruinosa* venoms ($P > 0.05$ each, using *t*-tests in comparison to the respective response before the addition of the venom). Hence, independent of the sex of the spider, all of the tested *M. pruinosa* venom concentrations lacked neurotoxic effects in this bioassay.

4. Discussion

The average venom yield per spider in female *M. pruinosa* was 32 times that of male *M. pruinosa*. Unfortunately, only two female *M. pruinosa* were available for milking, resulting in three milkings only (i.e. one milking for each female plus one repeat in one of the females about 4 weeks later), while 230 milkings of male spiders were carried out from 101 males. In *M. bradleyi*, females yielded about 6.5 times the amount of proteins than males. One explanation for the very low venom yield in male *M. pruinosa* could be the fact that due to the short life span of adult males, the venom was milked up to twice a week over several weeks; hence the venom could only be partially re-synthesized between those milkings. In support, other spiders take around 2 weeks to regenerate an empty venom gland (Kuhn-Nentwig et al., 2004) and frequent milking drastically reduces venom production (Kaire, 1963; Atkinson, 1981). On the other hand, a lower venom yield for males is common in spiders and might be mainly explained by the usually larger size of female spiders. The fact that males yield less venom than their female counterparts has already been reported for several species such as *Atrax robustus* (Wiener, 1959), *Loxosceles reclusa* (Mor-

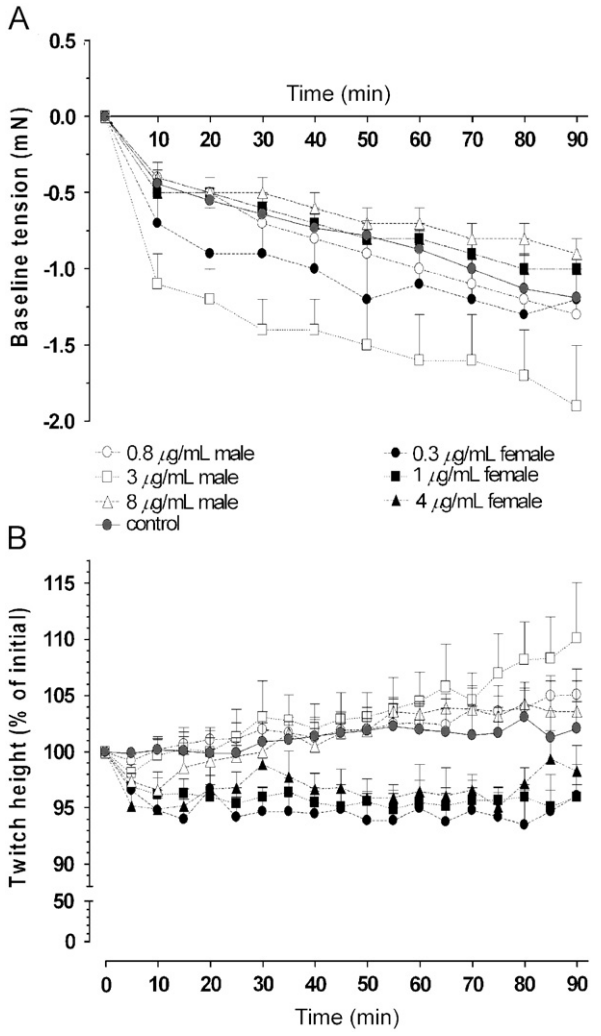

Fig. 5. Effect of *M. pruinosa* venom on the indirectly-stimulated chick biventer cervicis nerve–muscle preparation. All tested concentrations of male (open symbols) and female (closed symbols) *M. pruinosa* venom failed to significantly affect the (A) baseline muscle tension or (B) electrically stimulated twitch height. Note the break in the *y*-axis in panel (B).

gan, 1969; Morris and Russell, 1975), *Scodra griseipes* (Celerier et al., 1993), *L. intermedia* (De Oliveira et al., 1999), *Tegenaria agrestis* and *T. gigantea* (Binford, 2001), *Phoneutria nigiventer* (Herzig et al., 2002) and *Cupiennius salei* (Kuhn-Nentwig et al., 2004). Consequently, the sexual dimorphism that is found in most adult spiders is not specific to morphological characteristics, but can also be manifested as marked differences in the venom yield.

Due to methodological differences (e.g. differences in the software and peak detection settings, the columns used and the applied acetonitrile gradient) in HPLC separation of *M. pruinosa* and *M. bradleyi* venoms, it was impossible to directly compare the venom compositions between these species. This might explain why, despite a more complex appearance (Fig. 1), the analysis of the HPLC chromatograms resulted in a lower number of peaks for *M. bradleyi* venoms (independent of the sex of the spiders). Nevertheless, the chromatograms for each species were analysed separately for intersexual differences in venom composition. In both species, the number of major peaks was higher in female than in male venoms and about half of the peaks were present in the venoms of both sexes, with the remaining peaks being either specific for male or female venoms. Large quantitative intersexual differences (i.e. more that 10% difference in peak area) were observed in three peaks in *M. pruinosa* venom and in six peaks in *M. bradleyi* venom. It is difficult to draw a clear conclusion from the analysis of the HPLC chromatograms, since a similar retention time does not necessarily imply that the same toxins are present in this fraction. Hence, the observed intersexual differences in venom composition could be either qualitative or quantitative or both. However, the considerable intersexual differences in these *Missulena* venoms observed by HPLC might suggest intersexual variations in (at least) some bioactivities of these venoms.

The MALDI-TOF mass spectra show that the molecular masses in the venoms of both *Missulena* species are within a range of 3000–8000 m/z, a feature now well defined for spider venoms and shared by most mygalomorph venoms including Australian funnel-web spider venoms (see Escoubas and Rash, 2004; Escoubas et al., 2006). However, without further separation or sequence information it remains difficult to determine the similarity of venom components between both sexes. Comparison of both sexes across species does not reveal either striking differences in mass distribution or exact similarities, although it would be expected that both species may share some toxins, or that at least they would produce very homologous peptides with similar pharmacology. An example of the degree of intersexual variation in the composition of mygalomorph spider venoms was recently given by Mourão et al. (2007). They reported 125 (male) and 195 (female) molecular masses from the venom of the South-American theraphosid spider *Acanthoscurria atrox*, but only 35 masses were common in both sexes. The fact that neither δ- nor ω-missulenatoxin-Mb1a (Gunning et al., 2003;

Chong et al., unpublished data) could be detected at respectively m/z 4934.8 and 4167.5 in the mass spectrum of crude *M. bradleyi* venom could be due either to variation in venom composition between batches or more likely to ion suppression in crude venom, those toxins being minor components.

The KD_{50} and LD_{50} values indicate considerably higher interspecies than intersexual differences in the insecticidal properties of *Missulena* venoms in crickets. Within both *Missulena* species at 24–72 h post-injection, the difference in the respective KD_{50} or LD_{50} values between both sexes varied only in a small range (factor 0.7–1.6 in *M. pruinosa* and 1.2–2.6 in M. *bradleyi*). In contrast, the range for interspecies variation was considerably larger (factor 4.4–19.8 in male and 8.7–19.3 in female venom), with *M. bradleyi* venoms being more potent. It would appear that, independent of the sex of the spiders, *M. bradleyi* venom is more potent in crickets than *M. pruinosa* venom.

Our results for *M. bradleyi* venom in the indirectly stimulated chick biventer muscle parallel those published by Rash et al. (2000). Only male venom showed an activity, consisting of a large muscle contraction with fasciculation and a decrease in twitch height. These effects could be reversed by the addition of Sydney funnel-web spider antivenom. This is most likely the result of sequence similarities between δ-missulenatoxin-Mb1a (the active toxin in male *M. bradleyi* venom; Gunning et al., 2003) and δ-atracotoxin-Ar1a (the lethal neurotoxin from the male Sydney funnel-web spider *A. robustus*; Nicholson et al., 1998) that are the epitopes for the antivenom. Venom of both sexes of *M. pruinosa* failed to produce significant effects on the baseline or twitch height in the chick biventer. There could be two reasons for the lack of neurotoxicity of *M. pruinosa* venom from both sexes, one being a complete absence of orthologs of δ-missulenatoxin-Mb1a in these venoms. Another reason could be that such orthologs are present, but in a very low concentration which does not produce significant neurotoxicity unless very high concentrations of the crude venom are used.

In summary, we found intersexual differences in *Missulena* venoms with respect to venom yield, composition and biological activity. In general, female *Missulena* yield more venom than their male counterparts. The present HPLC and mass spectrometry data show a substantial degree of intersexual variation in the venoms of *M. pruinosa* and *M. bradleyi*. In crickets, *M. bradleyi* venom was considerably more potent than *M. pruinosa* venom, while only small intersexual differences were observed for both *Missulena* species. In the chick biventer cervicis nerve–muscle bioassay, male (but not female) *M. bradleyi* venom induced large muscle contraction with fasciculation and decreased twitch height that could be reversed by the addition of funnel-web spider antivenom. Venoms of both sexes of *M. pruinosa* did not induce significant effects on neurotransmission in the chick biventer cervicis nerve–muscle preparation. We therefore assume that female *M. bradleyi* venom and venoms from male and female *M. pruinosa* do not contain orthologs of δ-missulenatoxin-Mb1a from male *M. bradleyi* venom (Gunning et al., 2003). Further tests of *M. pruinosa* venom in other vertebrate species are required to obtain more data on venom toxicity. Nevertheless, the overt lack of toxicity in a vertebrate nerve–muscle preparation is consistent with clinical reports that, apart from male *M. bradleyi*, mouse spider venoms are not a major medical problem in humans (Isbister, 2004).

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