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**Snake venoms as a source of compounds modulating sperm
physiology: secreted phospholipases A₂ from *Oxyuranus
scutellatus scutellatus* impact sperm motility, acrosome reaction
and *in vitro* fertilization in mice**

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ABSTRACT

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5 The goal of this study was to identify new compounds from venoms able to modulate sperm
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7 physiology and more particularly sperm motility. For this purpose, we screened the effects of
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9 16 snake venoms cleared of molecules higher than 15 kDa on sperm motility. Venoms rich in
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11 neurotoxins like those from *Oxyuranus scutellatus scutellatus* or *Daboia russellii*, were highly
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13 potent inhibitors of sperm motility. In contrast, venoms rich in myotoxins like those from
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15 *Echis carinatus*, *Bothrops alternatus* and *Macrovipera lebetina*, were inactive. From the main
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17 pharmacologically-active fraction of the Taipan snake *Oxyuranus scutellatus s.*, a proteomic
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19 approach allowed us to identify 16 different proteins, among which OS1 and OS2, two
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21 secreted phospholipases A2 (sPLA₂). Purified OS1 and OS2 mimicked the inhibitory effect on
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23 sperm motility and were likely responsible for the inhibitory effect of the active fraction. OS1
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25 and OS2 triggered sperm acrosome reaction and induced lipid rearrangements of the plasma
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27 membrane. The catalytic activity of OS2 was required to modulate sperm physiology since
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29 catalytically inactive mutants had no effect. Finally, sperm treated with OS2 were less
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31 competent than control sperm to initiate *in vitro* normal embryo development. This is the first
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33 report characterizing sPLA₂ toxins that modulate *in vitro* sperm physiology.
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1. Introduction

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5 Human fecundity has been decreasing for several decades and it is becoming a major
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7 problem in modern society [1]. This decline is first likely due to a societal cause, related to the
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9 postponed decision of first pregnancy, and second to an environmental cause related to the
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11 increased levels of pollutants, and in particular of endocrine disruptors which might interfere
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13 directly with gametogenesis [2]. Infertility treatment is hampered by our incomplete
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15 knowledge of sperm physiology and fertilization, especially at the molecular level. To unravel
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17 the mechanisms underlying the reduction of reproductive fitness, one can identify the genes
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19 involved in gametogenesis through the study of infertile couples and animals models [3-5] or
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21 analyze the effects of various compounds having specific functions. The molecular pathways
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23 and the different proteins involved in the three main physiological functions of mature sperm
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25 occurring in the female tract that are sperm motility, capacitation and acrosome reaction are
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27 still subjects of intensive research [6, 7]. Discovering new molecular pathways and/or proteins
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29 involved in the control of sperm physiology is a necessity. Different strategies including gene
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31 targeting and proteomic analyses have been used to identify the molecular mechanisms of
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33 sperm physiology like invalidating genes or performing proteomic analyses. Another strategy
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35 is based on the use of very specific inhibitors of a sperm function, and the purification of the
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37 targeted protein by using the inhibitor as a bait. This strategy has been very successful in the
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39 ion channels field. For instance, the plant alkaloid ryanodine allowed the characterization of a
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41 Ca^{2+} channel from the endoplasmic reticulum while dihydropyridines allowed the
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43 characterization of another Ca^{2+} channel from T tubules of the skeletal fibers [8]. In sperm, a
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45 similar approach using a specific sperm antibody allowed the characterization of Izumo, a
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47 unique protein involved in sperm-oocyte binding [9].
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Venoms have also been used as a major source of biological active molecules to provide key tools to purify and characterize new mammalian proteins. For instance, apamin from bee venom has allowed to characterize a Ca^{2+} -activated K^+ -channel [10]. Several types of molecules are present in venoms, including proteins, peptides and polyamines, and more than hundred compounds can be found in a single venom. Most venom compounds are highly specific. Such high specificity can be illustrated by the property of the spider toxin ω -AgaIVA that distinguishes between two spliced variants of Ca^{2+} channels. Indeed, ω -AgaIVA inhibits P-type but not Q-type Ca^{2+} channels, while these channels result from the alternative splicing of the *Cacna1a* gene [11]. The specificity of venom compounds is also illustrated by the fact that venom toxins or compounds structurally close to them are used as drugs in the treatment of pain, neurological diseases, cancers or autoimmune diseases [12]. Because of the diversity and specificity of venom compounds, we undertook a wide screening of various snake venoms in order to characterize new biologically active compounds acting on sperm functions.

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In this paper, we screened on sperm motility 16 different snake venoms belonging to *Elapidae*, *Viperidae* and *Colubridae* families. Crude venoms were first fractionated by gel filtration to remove proteases, and fractions with molecular masses lower than 15 kDa were tested. Most venoms were potent inhibitors of sperm motility except those from *Echis carinatus*, *Bothrops alternatus* and *Macrovipera lebetina*. The two most potent venoms were those from *Oxyuranus scutellatus scutellatus* and *Daboia russellii*. We then focused on the most potent inhibiting fraction from *Oxyuranus scutellatus scutellatus*. By a proteomic analysis, we demonstrated that this fraction is enriched in secreted phospholipases A_2 (sPLA₂), in particular OS1 and OS2, two snake sPLA₂ with different toxicological and pharmacological properties [13]. We thus tested the effects of recombinant OS1, OS2 as well as of mutant proteins having different levels of enzymatic activities on sperm motility, sperm

1 acrosome reaction (AR), and *in vitro* fertilization (IVF). We found that recombinant OS1 and
2 OS2 sPLA₂s were potent inhibitors of sperm motility while they were potent activators of
3 acrosome reaction. *In vitro* fertilization experiments indicate that embryos obtained with OS2-
4 treated sperm showed an impaired development, suggesting that sperm lipid metabolism is
5 important for the fertilizing potential of sperm.
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2. Materials and methods

2.1. Venom separation

Venoms were obtained from Latoxan, SA (Valence, France). Hundred mg of crude venom was dissolved in 1 ml of 100 mM ammonium acetate pH 6.8 and centrifugated at 10,000 g for 10 min. Two hundred fifty μ l of supernatant was used for purification by gel filtration. Separation was performed on a SuperdexTM peptide 10/300 GL gel filtration column with elution at 0.5 ml/min in 100 mM ammonium acetate pH 6.8. Fractions of 500 μ L were frozen after collection before subsequent assays. Fractions with molecular masses higher than 15 kDa were discarded (fraction A1 to A8). For screening, we first pooled fractions A9-B4 to test the overall activity. The pooled fractions were prepared by mixing 100 μ L of fractions A9 to B4, followed by lyophilization and solubilization in 120 μ L of M16 medium.

2.2. Computer-assisted motility analysis

All protocols have been reviewed and approved by the local ethical committee. OF1 male mice were 2-6 months old (Charles River, France). Sperm from caudae epididymides were allowed to swim in 1 ml of M2 medium for 10 min. Sperm was resuspended in M16 and incubated with venom fractions at 37°C for 10 min (237.5 μ L of sperm were mixed with 12.5 μ L of a fraction). After incubation, the sperm suspension was immediately placed onto an analysis chamber (2X-CEL Slides, 100 μ m depth, Leja Products B.V., Netherlands) and kept at 37 °C for microscopic quantitative study of sperm movement. Sperm motility parameters were measured at 37°C using a sperm analyzer (Hamilton Thorn Research, Beverley). The settings employed for analysis were as follows: acquisition rate: 60 Hz; number of frames: 100; minimum contrast: 25; minimum cell size: 10; low static-size gate: 2.4; high static-size gate: 2.4; low static-intensity gate: 1.02; high static-intensity gate: 1.37 ; minimum elongation

gate: 12; maximum elongation gate: 100; magnification factor: 0.70. The motility parameters measured were curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH). A minimum of 100 motile spermatozoa were analyzed for each assay.

2.3. Proteomic analysis

In order to increase the sequence coverage of identified proteins, three experiments were performed with two distinct chromatographic gradients and three methods of mass spectrometry. Protein samples (experiment 1 and 2: 21 μ L, experiment 3: 10 μ L) were dried by vacuum centrifugation and resuspended in 50 mM ammonium bicarbonate (pH 8), denatured with 2% sodium dodecyl sulfate, reduced with 45 mM dithiothreitol (50°C for 30 min) and alkylated with 100 mM iodoacetamide at room temperature for 40 min. The protein mixture was then digested by addition of a trypsin solution (Porcine modified, Promega) at 1:20 (experiments 1 and 2) or 3:20 (experiment 3) ratio (w/w), and incubated overnight at 37°C. Protein digests were dried by vacuum centrifugation and resuspended in 20 μ L of buffer A (2% acetonitrile in water with 0.05% TFA). Three μ L of the resulting sample were first loaded onto a trap column (PepMap100 C18, 5 μ m, 100 \AA , 300 μ m i.d. x 5 mm, Dionex Corporation) for desalting and concentration, and then back-eluted onto the column (PepMap100 C18, 3 μ m, 100 \AA , 75 μ m i.d. x 15 cm, Dionex Corporation). The flow rate through the column was set to 300 nL/min. Mobile phase buffers were: A = 2% acetonitrile in water with 0.05% TFA and B = 80% acetonitrile in water with 0.04% TFA. Peptides were separated using the following gradient: (i) experiments 1 and 3: 0-5 min: 0-10% B (v/v), 5-45 min: 10-42% B (v/v), 45-55 min: 42-58% B (v/v), 55-65 min 90% B (v/v) and then re-equilibrated :65-80 min 100% A (v/v); (ii) experiment 2: 0-5 min: 0-10% B (v/v), 5-40 min 10-42% B (v/v), 40-55 min 42-90% B (v/v), 55-70 min 90% B (v/v) and then re-equilibrated :70-85 min 100% A (v/v). The eluent was spotted directly onto a MALDI target plate

1 (Applied Biosystems) using a Probot Microfraction Collector (Dionex Corporation). The
2 eluent was mixed 1:3 (v/v) post-column with 2 mg/mL HCCA in 70% acetonitrile and 0.1%
3 TFA. Mass spectrometry analyses were performed on a 4800 MALDI-TOF/TOF instrument
4 (Applied Biosystems). The instrument was operated in positive ion mode and externally
5 calibrated. MS spectra were acquired over a mass range of 750-3500 m/z. For each sample
6 spot, a maximum of 20 precursors with S/N over 50 were selected for fragmentation, starting
7 with the strongest (experiments 1 and 3) or weakest (experiment 2) precursor. MS/MS spectra
8 were collected at the most intense point of the chromatographic elution profile, using air as
9 the collision gas and a collision energy of 1 kV. For protein identification, all MS/MS data
10 obtained (experiments 1, 2 and 3) were submitted to Protein Pilot 2.0.1 software program
11 (Applied Biosystems) using the Paragon protein database search algorithm [14], with trypsin
12 as the digestion agent, cysteine modification by iodoacetamide and Rapid ID as search effort.
13 Searches were performed against the NCBI protein database (update 05-10-2009), limited to
14 *Oxyuranus scutellatus* sequences. Only proteins identified with at least a confidence of 95%,
15 or a ProtScore of 1.3, were reported.
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39 2.4. Production of recombinant sPLA₂

40 Native OS1 and OS2 sPLA₂s from *Oxyuranus scutellatus scutellatus* (Taipan) snake venom
41 and native Ba-II and Ba-IV K-49 sPLA₂-like proteins from *Bothrops asper* venom were
42 purified as described [15, 16]. Recombinant wild-type OS2 and its catalytically-inactive
43 mutants were prepared and purified to homogeneity as described previously [13]. We did not
44 observe any potency differences between native and recombinant enzymes and results
45 obtained with both types of protein were pooled.
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58 2.5. Acrosome reaction assay

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1 Sperm from caudae epididymes of OF1 mice were allowed to swim in M2 medium for 10
2 min. For sPLA₂ treatment, sperm were incubated with sPLA₂ in M16 medium at 37°C for the
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4 last 10 min. Sperm were then transferred to PBS solution and fixed with 4%
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6 paraformaldehyde for 2 min. Sperm were washed with 100 mM ammonium acetate pH 6.8 for
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8 2 min, wet-mounted on slides and allowed to air dry. Slides were rinsed with water, stained
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10 with Coomassie blue (0.22%) for 2 min and finally rinsed with water. Slides were counted
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12 immediately and at least 150 sperm cells were scored per slide.
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19 *2.6. Confocal microscopy*

21 Control untreated sperm (non capacitated) or sperm treated for 10 min with 200 nM OS2 in
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23 M16 medium were washed twice with PBS, and then incubated in a staining buffer (from
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25 Apoptosis detection kit-Sigma Aldrich, France) containing both carboxyfluorescein diacetate
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27 (6-CFDA) and 1 µg/mL annexin V-Cy3 for 15 min in the dark at room temperature. After
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29 incubation with annexin V-Cy3, sperm was washed twice with the staining buffer and fixed in
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31 4% paraformaldehyde. Annexin-V staining was analyzed using a confocal microscope
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33 (Leica).
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41 *2.7. Chemical compounds*

42 M2, M16 medium and Apoptosis detection kit were purchased from Sigma.
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48 *2.8. Statistical analyses*

49 Statistical analyses were performed with SigmaPlot. T-test and paired t-test were used to
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51 compare the effects of various compounds on AR and fertility, respectively. Data represent
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53 mean ± SEM. Statistical tests with a p values ≤ 0.05 were considered as statistically
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55 significant.
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3. Results

3.1 Venom sPLA₂s inhibit sperm motility

Our main goal was to identify compounds from venoms able to modulate sperm physiology and more particularly sperm motility. We used a CASA system (Computer Assisted Sperm Analysis) to test the effects of venom fractions on sperm motility. We focused more particularly on two sperm motility parameters, the VCL corresponding to Curvilinear Velocity (microns/sec) and the ALH corresponding to Amplitude of Lateral Head Displacement (microns). We tested on sperm motility 16 different snake venoms from *Elapidae*, *Crotalidae* and *Colubridae* families that originate from different continents (South and North America, Asia and Africa). The venoms were first fractionated by size exclusion chromatography and fractions containing large proteins such as proteases were discarded (Fig. 1A). Fractions containing proteins higher than 15 kDa were identified by calibrating the gel filtration column with protein standards (cytochrome C, aprotinin and vitamin B12). A gel analysis with Coomassie staining was performed to check that proteins above 15 kDa were absent from the fractions of interest (not shown). Second, a two-step screening protocol was developed to test the venom fractions. First, we tested all collected fractions below 15 kDa from one venom as a single pool. Second, we focused on the most inhibiting venoms and tested individually the different collected fractions. Fig. 1B presents the effects of the 16 tested venoms on non capacitated sperm incubated during 10 min with the pooled fractions (A9-B4) from the different venoms. *Daboia russellii* and *Oxyuranus scutellatus scutellatus* venoms were particularly potent and decreased sperm VCL by around 50%. On the other hand, *Echis carinatus*, *Bothrops alternatus* and *Macrovipera lebetina* venoms were inactive. The other venoms presented intermediate activities on sperm motility. To identify the compounds involved in the decrease of motility, we then tested individually the fractions from

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Oxyuranus scutellatus scutellatus. Fraction A9 consisting of compounds with the highest molecular masses (10-15 kDa) was the most potent and decreased sperm VCL by more than 30% (Fig. 1C). We then analyzed the composition of fraction A9 by a proteomic approach. Sixteen non-redundant proteins were identified with a confidence of at least 95% (Table 1). Redundancy between proteins identified was minimized using a grouping function, which assigned an "unused score" to the peptides that are unique to a protein or group of redundant proteins. For example, OS2 is a competitor protein of PLA-7 precursor because it contains nearly all the peptides of PLA-7 precursor but has no peptides distinct from this latter. Similarly, OS1 is a competitor protein of PLA-1 precursor / OS5 precursor. Interestingly, secreted phospholipases A2 (sPLA₂s) and related proteins were highly represented in the identified proteins and constituted good candidates. We focused on two proteins having the highest number of identified peptides, *i.e.* OS1 and OS2 sPLA₂s (Table 1) and tested their activities on sperm motility by using native and recombinant enzymes. A 10 min incubation with OS1 and OS2 at 200 nM modified the head sperm tracks recorded by the CASA system (Fig. 2A). The two sperm motility parameters VCL and ALH were decreased by both sPLA₂s (Fig. 2B-C), with OS2 being more potent than OS1. Interestingly, concentrations of OS2 as low as 0.2 nM were able to modify sperm VCL.

3.2. sPLA₂ catalytic activity is required for venom sPLA₂ effects on sperm motility

Snake venom sPLA₂s are structurally-conserved small enzymes with a low molecular mass of 13-18 kDa and are rich in disulfide bonds [17-19]. Snake venom sPLA₂s are divided into catalytically active and catalytically inactive enzymes. sPLA₂s bearing aspartate at position 49 (D49-sPLA₂s) are highly catalytically active whereas K49, S49- or N49-sPLA₂s present a low or no catalytic activity [20]. If sPLA₂ enzymes are first characterized by their ability to hydrolyze the *sn*-2 ester bond of glycerophospholipids to produce fatty acids and

1 lysophospholipids [21], they also act as agonists for specific lectin receptors and other
2 proteins [22, 23]. Concerning neurotoxic sPLA₂, their mechanism of action is still an open
3 question: some reports highlight the role of sPLA₂ metabolites, that are fatty acids and
4 lysophospholipids [24] whereas others involve binding to proteins [13, 22]. Because several
5 lectins receptors are present in the sperm membrane [25], we wondered which mechanism of
6 action is involved in the effect of sPLA₂ on sperm motility. To investigate this question, we
7 tested a H48Q catalytic mutant of OS2 which has a 500-fold lower catalytic activity than the
8 wild-type enzyme [13, 26]. The H48Q OS2 mutant was inactive on sperm VCL (Fig. 3A). We
9 also tested a chimeric OS2/OS1 sPLA₂ where the OS2 C-terminal domain (AA 109-128) has
10 been substituted by the C-terminal domain of OS1. This OS2/OS1 chimera has a 500-fold
11 lower enzymatic activity but interestingly retains most of the neurotoxicity of wild-type OS2
12 [13]. The chimera was also unable to inhibit sperm motility.

13 The above screening experiments have shown that venoms from *Echis carinatus*, *Bothrops*
14 *alternatus* and *Macrovipera lebetina* were inactive. The two first venoms are characterized by
15 the presence of a high level of sPLA₂ myotoxins [16, 27, 28], characterized by a mutation at
16 amino acid 49 and leading to a strong decrease of its catalytic activity. We tested the purified
17 sPLA₂-like myotoxins BaII-K49 and BaIV-K49 from the venom of *Bothrops asper* and found
18 that both proteins were fully inactive on sperm motility (Fig. 3B). In good accordance, we
19 found that a D49K mutant of OS2, which has no detectable enzymatic [13], was also inactive
20 on sperm motility (Fig. 3B). All together, these results indicate the importance of the catalytic
21 activity of OS1 and OS2 in decreasing sperm motility.

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3.3. OS2 treatment does not impair sperm viability, but converts the fast-speed sperm population into the low-speed sperm population

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Venoms are known to produce tissue lysis and cell death. We thus addressed whether the effect of OS2 on sperm motility was due to sperm lysis and death. We first measured the speed distribution of sperm treated with OS2 versus control (Fig. 4A). The distribution of OS2-treated sperm was characterized by the absence of fast sperm, i.e. sperm with a VCL above 200 $\mu\text{m/s}$, and by an over-representation of the slow sperm subpopulation, i.e. sperm with a VCL between 50-100 $\mu\text{m/s}$. On the other hand, OS2 treatment did not increase the immotile subpopulation (0-10 $\mu\text{m/s}$). The fact that most of the treated cells were still motile after treatment suggests that OS2 did not induce a fast cell death responsible for the measured effect on sperm motility, but rather converted the fast-speed sperm into low-speed ones. To confirm this result, we loaded OS2-treated sperm with 6-carboxyfluorescein diacetate (6-CFDA), a compound which is used as a marker of sperm viability and enters freely into living cells to produce green fluorescence after de-esterification by endogenous esterases [29]. OS2-treated sperm subsequently incubated with 6-CFDA presented a strong green fluorescence (Fig. 4B), indicating that OS2 treatment did not induce cell death. Finally, we measured phosphatidylserine staining with annexin-Cy3 before and after OS2 treatment, as a marker of changes in lipid asymmetry at the plasma membrane during sPLA₂ treatment. It is important to point out that in sperm cells, externalization of phosphatidylserine occurs naturally during capacitation and is not considered as a hint of apoptosis [30]. Interestingly, a 10 min treatment of non-capacitated sperm with OS2 induced a strong externalization of phosphatidylserine at the plasma membrane (Fig. 4C). All together, these results suggest that OS2 treatment produces a lipid reorganization of the plasma membrane similar to that occurring during capacitation and without inducing any obvious sperm cell death.

3.4. OS1 and OS2 are very potent modulators of acrosome reaction and fertilization

1 Different studies have shown that mammalian PLA₂s play important roles in capacitation,
2 acrosome reaction (AR) and the early steps of fertilization including sperm binding and
3 sperm-oocyte fusion [31]. AR induced by non physiological and physiological stimuli (Ca²⁺
4 ionophore, progesterone and zona pellucida) is prevented by PLA₂ inhibitors [32-35] and
5 produce the release of lipid metabolites like fatty acids and lysophosphatidylcholine (LPC).
6 Moreover, LPC and fatty acids accelerate or promote exocytosis [36, 37]. Finally, LPC
7 improves sperm binding on zona pellucida [38] and sperm-oocyte fusion [39]. This
8 physiological context prompted us to test the effects of OS1 and OS2 on AR and fertilization.

9 We thus tested the potency of OS1 and OS2 to induce AR. Dose-response curves showed that
10 OS2 is more potent than OS1 to induce AR (Fig. 5). Importantly, only a fraction of the sperm
11 subpopulation was sensitive to the sPLA₂. Indeed, concentrations above 100 nM did not
12 further produce AR of the treated sperm, and about 40% of the sperm population appear to be
13 resistant to OS2 treatment. This result may be explained by the presence of two sperm
14 populations with a different sensitivity to OS2 and fits with earlier findings indicating that the
15 sperm plasma membrane composition is heterogeneous [40].

16 We have shown that OS1 and OS2 are potent modulators of sperm physiology, inhibiting
17 sperm motility, triggering sperm AR and producing lipid reorganization of the plasma
18 membrane. We next evaluated the impact of such modifications on the sperm fertilization
19 potential. To address this question, we compared *in vitro* fertilization outcomes obtained with
20 control and OS2-treated sperm. We thus treated capacitated sperm briefly with 20 or 200 nM
21 OS2 during 10 min before mixing sperm and oocytes for fertilization (Fig. 6A). Twenty four
22 hours after fertilization, the rate of oocytes reaching the 2-cell embryo stage was counted and
23 compared to IVF performed with untreated sperm and oocytes from the same batches (Fig.
24 6B). OS2 decreased the IVF outcomes in a dose-dependent manner (Fig. 6C). It is important
25 to note that the fertilization rate is not modified for OS2-treated sperm. On the other hand,
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embryos obtained with OS2-treated sperm presented a defect in the first step of development, corresponding to the first cleavage, as illustrated by the increased rate of aborted embryos.

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4. Discussion

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2 In this manuscript, we have shown that fractions from various snake venoms
3 containing molecules below 15 kDa can inhibit sperm motility. Moreover, we found that the
4 inhibiting activity from the venom of *Oxyuranus scutellatus scutellatus* on sperm motility is
5 mostly due to compounds with a molecular mass around 10-15 kDa. By using a proteomic
6 approach and purified toxins, we found that the effects of the active fraction were mostly due
7 to the sPLA₂ OS1 and OS2. Indeed, recombinant OS1 and OS2 sPLA₂s inhibited sperm
8 motility. Moreover, OS1 and OS2 activities on sperm motility were due to their enzymatic
9 activity on phospholipids, since several types of enzymatically inactive mutants were unable
10 to modulate sperm motility. Moreover, several snake venoms and more particularly those
11 from *Echis carinatus*, *Bothrops alternatus* and *Macrovipera lebetina* were unable to modulate
12 sperm motility. These venoms are rich in sPLA₂ myotoxins, which are characterized by a
13 D49K mutation, leading to complete loss of enzymatic activity. In agreement, two purified
14 sPLA₂-like myotoxins, BaII-K49 and BaIV-K49 that originate from the venom of *Bothrops*
15 *asper*, were fully inactive on sperm motility. All together, these results demonstrate that the
16 potency of a snake venom to decrease sperm motility is dependent on the catalytic activity of
17 the sPLA₂s present in the venom. However, possible other inhibiting or activating effects of
18 non-sPLA₂ compounds present in the venom may be masked by our screening procedure
19 using pooled fractions and the strong inhibitory effects of sPLA₂ on sperm motility.
20 Compounds with activating effects appeared to be present as suggested by the activating
21 effect of fraction B1 of *Oxyuranus scutellatus s.*

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51 The different secreted PLA₂s have been classified into ten different groups, namely I,
52 II, III, V, IX, X, XI, XII, XIII and XIV sPLA₂ [41]. A large variety of sPLA₂ are found in
53 venom of different animals such as insect (*Hymenoptera*), scorpions and snakes. Toxic sPLA₂
54 belong to three different groups: sPLA₂ from *Elapidae* and *Hydrophidae* snakes belong to

1 group IA, sPLA₂ from *Crotalidae* and *Viperidae* snakes belong to group IIA while sPLA₂
2 from *Hymenoptera* belong to group III. We did not observe a strong relationship between
3 sperm activity and sPLA₂ group classification since both OS2 (group IA) and OS1 (group IB ;
4 OS1 does belong to group IB since a typical pancreatic loop is present [42]) from *Oxyuranus*
5 *scutellatus* and likely sPLA₂ from *Daboia russelli* (group IIA) were all active. The molecular
6 diversity of snake venom sPLA₂ is particularly high and a single snake venom can contain up
7 to 15 different sPLA₂, with different patterns of enzymatic activities and toxicities [17]. As
8 also suggested by the different potencies of the snake venoms screened in this study, we
9 observed that two different sPLA₂s from *Oxyuranus scutellatus scutellatus*, OS1 and OS2,
10 presented marked differences in their ability to inhibit sperm motility, OS2 being about 10-
11 fold more potent than OS1. Recently, a toxin complex called “reprotoxin” and containing an
12 sPLA₂ subunit was found to induce atrophy of testis when injected at a sub-lethal dose [43].
13 Our results show that sperm membrane composition are deeply affected by sPLA₂ activity and
14 may eventually lead to cell apoptosis. Our results may thus explain the testis atrophy induced
15 by reprotoxin.

16 This study further confirms that changing the sperm plasma membrane composition, and more
17 particularly phospholipids via sPLA₂ enzymatic activity, has important physiological
18 consequences in terms of sperm motility, capacitation and acrosome reaction. It is important
19 to point out that the strong decrease of sperm motility was not due to a loss of cell viability
20 since OS2-treated sperm was still able to hydrolyze the fluorescent probe by non-specific
21 esterases. The decrease of sperm motility was likely due to changes in the composition of the
22 plasma membrane, as demonstrated by the change in annexin-V staining after OS2 treatment.
23 This hypothesis is also supported by the fact that human astenozoospermia (sperm with low
24 motility) is associated with a defective sperm plasma membrane composition with an excess
25 of cholesterol and desmosterol [44, 45], a low concentration of specific fatty acids like
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1 docosaehaenoic acid found in different phospholipids [46] and an overall increase of fatty
2 acid content [47, 48]. These results suggest that lipid rearrangement or lipid metabolites are
3 important for flagellum beat, in order either to change membrane fluidity or to control key
4 proteins involved in flagellum beat. Venom sPLA₂s, by changing lipids of the plasma
5 membrane, are likely to mimic the action of endogenous mammalian sPLA₂s, known to
6 control sperm motility. For instance gene knock-out of the intracellular PLA₂ VIA (iPLA₂β)
7 leads to a severe motility phenotype [49]. Overall, our results highlight the importance of
8 sPLA₂ as a modulator of sperm physiology. Indeed, mammalian sPLA₂s are widely expressed
9 in male reproductive tissues [50] and are likely to be involved in a large set of biological
10 functions that remain to be determined.

24 OS1 and OS2 were also potent inducers of acrosome reaction, with marked effects at
25 concentrations as low as 0.2 nM for OS2. Interestingly, the potent OS2 sPLA₂ was unable to
26 trigger AR in the whole sperm population even at very high concentrations, and about 30-40%
27 of sperm were fully resistant. This point highlights the heterogeneity of the sperm population
28 [40, 47]. Finally, the fact that embryos obtained with OS2-treated sperm presented an
29 impaired development during the first cleavage stresses the importance of the quality of the
30 sperm plasma membrane composition and integrity for embryo development [51]. This result
31 points out to the importance of sperm phospholipids in the first steps of embryo development.

43 In conclusion, our results highlight two important characteristics of mammalian sperm
44 phospholipids during fertilization that include their heterogeneity and their role in embryo
45 development. This point suggests that the lipid composition of the sperm should be taken into
46 account in assisted reproductive techniques like ICSI (Intra Cytoplasmic Sperm Injection),
47 where the physiological sperm sorting mechanisms, which are normally occurring in the
48 female tract, are not reproduced [52].

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Table/Figures legends

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3 **Table 1:** list of identified proteins in the fraction 9 of *Oxyuranus scutellatus*. N: rank of the
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5 specified protein relative to all other proteins in the list of detected proteins. Unused
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7 (ProtScore): measure of the protein confidence for a detected protein, calculated from the
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9 peptide confidence for peptides from spectra that have not been completely "used" by higher
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11 scoring winning proteins. Total (ProtScore): measure of the total amount of evidence for a
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13 detected protein. The Total ProtScore is calculated using all the peptides detected for the
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15 protein. % Cov (% Coverage): number of amino acids matching to at least one identified
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17 peptide divided by the total number of amino acids in the protein sequence, expressed as a
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19 percentage.
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27 **Fig. 1:** Snake venoms modulate sperm motility. (A) Venoms were separated by size exclusion
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29 chromatography on a SuperdexTM peptide 10/300 GL. Fractions containing molecules above
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31 15 kDa (A1-A8) were discarded and not tested. Example of separation for the crude venom of
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33 *Oxyuranus scutellatus scutellatus* (blue curve). A calibration run using cytochrome C (12.3
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35 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.8 kDa) is presented (red curve). (B) Global
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37 activity test of 16 different venoms on sperm motility. Aliquotes of fractions A9-B4 were
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39 pooled and tested as a single pooled fraction for their global activity on sperm VCL. (C)
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41 Effect of the different fractions (A9-B3) of *Oxyuranus scutellatus scutellatus* on sperm
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43 motility.
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51 **Fig. 2:** OS1 and OS2, two sPLA₂s from the venom of *Oxyuranus scutellatus scutellatus*
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53 inhibit sperm motility. (A) Tracks of the head position of non capacitated sperm measured by
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55 the CASA system in three conditions: control (non treated) sperm, sperm treated with OS1
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1 sPLA₂ and sperm treated with OS2 sPLA₂. (B) Dose-response curves corresponding to the
2 inhibition of VCL by OS1 and OS2. (C) Similar dose-response curves for ALH.
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9 **Fig. 3:** Inhibition of sperm motility by OS2 is dependent on its enzymatic activity.

10 (A) Comparison of the effects of OS1, OS2, OS2-H48Q (catalytically inactive mutant) and
11 OS2-Cter (catalytically inactive OS2-OS1 chimera) on sperm motility at 200 nM. (B)
12 Comparison of the effects of two different myotoxins (BaII-K49 and BaIV-K49 from the
13 venom of *Bothrops asper*) and OS2-D49K (a catalytically inactive mutant) on sperm motility.
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24 **Fig. 4:** OS2 is a potent activator of acrosome reaction. (A) Non capacitated control sperm:
25 most sperm are non-acrosome reacted and exhibit dark staining in the apical head (arrows).
26 (B) Sperm incubated for 10 min in the presence of 200 nM OS2: most sperm are acrosome
27 reacted, as indicated by the absence of a stained region in the apical head (black arrows). Note
28 that some sperm are non-acrosome reacted (white arrows) (C) Dose-response curves
29 corresponding to the acrosome reaction triggered by OS1 and OS2. Spontaneous AR
30 measured on untreated sperm was subtracted and the presented values correspond to sPLA₂-
31 induced AR.
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46 **Fig. 5:** OS2 does not induce a fast sperm death and triggers externalization of
47 phosphatidylserine. (A,B) Distribution of the different subpopulations of sperm as a function
48 of VCL or ALH values of non-treated control sperm (green bars), OS1-treated sperm (red
49 bars) and OS2-treated sperm (black bars). (C,D) Non-capacitated sperm incubated in the
50 presence of carboxyfluorescein diacetate (6-CFDA) for 15 min, after a 10 min pre-incubation
51 with control medium (C) or 200 nM OS2 (D). (E,F) Non-capacitated sperm incubated in the
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1 presence of annexin-V-cyanin-3 for 15 min, after a 10 min pre-incubation with control
2 medium (E) or 200 nM OS2 (F).
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7 **Fig. 6:** Impaired *in vitro* development of embryos obtained with OS2-treated sperm. (A)
8 Schematic drawing of a typical IVF experiment. Sperm were first capacitated for 35 min in
9 M16-2% BSA and then incubated for the last 10 min with M16 medium containing 20 nM or
10 200 nM OS2. After treatment, sperm were washed by centrifugation to remove unbound OS2,
11 putative catalytic products and all acrosomal compounds released during sPLA₂-induced AR.
12 Finally, washed sperm were introduced into droplets containing oocytes (20-85 oocytes per
13 experiment). After 4 h of gamete mixing, unbound sperm were washed away and IVF
14 outcomes were scored at 24 h. (B) Pictures of the different stages obtained after IVF at 24 h:
15 unfertilized oocytes; 2-cell embryos (normal development); aborted embryos corresponding to
16 oocytes with either multiple and uncontrolled divisions or presenting a second polar body
17 (PB) but no cell division. C. Outcomes of *in vitro* fertilization performed with either control
18 sperm or sperm briefly treated with 20 or 200 nM OS2.
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N	Unused	Total	%Cov	Accession	Name	Species
1	28,40	28,40	70,55	gi 71066730	PLA-7 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
1	0,00	26,40	84,03	gi 913014	OS2=secretory phospholipase A2 [Oxyuranus scutellatus=Taipan snakes, ssp. scutellatus, venom, Peptide, 119 aa]	Oxyuranus scutellatus
2	16,54	16,54	64,94	gi 71066720	PLA-1 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
2	0,00	16,54	64,94	gi 66475088	OS5 precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
2	0,00	16,00	73,23	gi 913013	OS1=secretory phospholipase A2 [Oxyuranus scutellatus=Taipan snakes, ssp. scutellatus, venom, Peptide, 127 aa]	Oxyuranus scutellatus
3	13,57	13,57	65,06	gi 71066802	SNTX-1 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
3	0,00	13,57	65,06	gi 123910874	RecName: Full=Short neurotoxin 1; Short=SNTX-1; AltName: Full=Toxin 3; Flags: Precursor	Oxyuranus scutellatus scutellatus
3	0,00	13,52	57,83	gi 66475096	toxin 3 [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
3	0,00	12,00	67,74	gi 254772678	RecName: Full=Short neurotoxin 2; Short=SNTX-2	Oxyuranus scutellatus scutellatus
4	10,69	10,69	91,94	gi 263546	taicatoxin serine protease inhibitor component [Oxyuranus scutellatus=Australian taipan snakes, ssp. scutellatus, venom, Peptide, 62 aa]	Oxyuranus scutellatus
5	10,00	14,00	57,93	gi 71066718	beta taipoxin variant 1 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
5	0,00	14,00	57,93	gi 66475082	beta taipoxin precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
5	0,00	14,00	71,19	gi 129435	RecName: Full=Phospholipase A2 homolog, taipoxin beta chain	Oxyuranus scutellatus scutellatus
6	10,00	14,00	44,52	gi 66475084	alpha taipoxin-2 precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
7	8,01	8,01	40,22	gi 254772668	RecName: Full=Long neurotoxin 1; Short=LNTX-1; Flags: Precursor	Oxyuranus scutellatus scutellatus
7	0,00	8,01	40,22	gi 118151706	LNTX-1 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
8	6,89	14,89	70,59	gi 129413	RecName: Full=Phospholipase A2, taipoxin alpha chain; AltName: Full=Phosphatidylcholine 2-acylhydrolase	Oxyuranus scutellatus scutellatus
9	6,00	26,00	69,86	gi 66475090	OS6 precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
9	0,00	24,00	68,49	gi 66475092	OS7 precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
10	6,00	10,00	56,82	gi 239977265	RecName: Full=Venom protease inhibitor 1; Flags: Precursor	Oxyuranus scutellatus scutellatus
10	0,00	10,00	56,82	gi 239938649	RecName: Full=Taicatoxin, serine protease inhibitor component; Short=TCX; Flags: Precursor	Oxyuranus scutellatus scutellatus
10	0,00	10,00	56,82	gi 185533608	taicatoxin serine protease inhibitor precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
10	0,00	10,00	56,82	gi 129919019	venom protease inhibitor precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
10	0,00	10,69	62,50	gi 239977272	RecName: Full=Venom protease inhibitor 2; Flags: Precursor	Oxyuranus scutellatus scutellatus
10	0,00	10,69	62,50	gi 129919043	venom protease inhibitor precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
11	4,96	4,96	75,00	gi 348613	alpha-neurotoxin-like taicatoxin component - Australian taipan (fragment)	Oxyuranus scutellatus scutellatus
11	0,00	4,96	75,00	gi 263548	taicatoxin alpha-neurotoxin-like component {N-terminal} [Oxyuranus scutellatus=Australian taipan snakes, ssp. scutellatus, venom, Peptide Partial, 28 aa]	Oxyuranus scutellatus
12	4,00	4,00	53,33	gi 71725729	venom natriuretic peptide OsNP-d precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
12	0,00	4,00	53,33	gi 123916490	RecName: Full=Natriuretic peptide OsNP-d; Flags: Precursor	Oxyuranus scutellatus scutellatus
12	0,00	4,00	29,73	gi 189047090	RecName: Full=Peptide TNP-b; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPb; Flags: Precursor	Oxyuranus scutellatus scutellatus
12	0,00	2,00	88,57	gi 32363244	RecName: Full=Peptide TNP-b; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPb	Oxyuranus scutellatus canni
12	0,00	2,00	88,57	gi 32363241	RecName: Full=Peptide TNP-a; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPa	Oxyuranus scutellatus canni
12	0,00	2,00	88,57	gi 32363240	RecName: Full=Peptide TNP-a; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPa	Oxyuranus scutellatus scutellatus
12	0,00	2,00	28,21	gi 32363246	RecName: Full=Peptide TNP-c; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPc	Oxyuranus scutellatus canni
13	4,00	4,00	37,35	gi 239977119	RecName: Full=Scutellin-3; Flags: Precursor	Oxyuranus scutellatus scutellatus
13	0,00	4,00	37,35	gi 185534290	scutellin-3 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
13	0,00	4,00	37,35	gi 157683297	scutellin-3 precursor [Oxyuranus scutellatus]	Oxyuranus scutellatus
14	2,00	4,00	29,73	gi 66475094	natriuretic peptide [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
14	0,00	4,00	29,73	gi 189047090	RecName: Full=Peptide TNP-b; AltName: Full=Taipan natriuretic peptide; AltName: Full=Venom natriuretic peptide OxsSNPb; Flags: Precursor	Oxyuranus scutellatus scutellatus
15	2,00	4,00	12,50	gi 66475086	gamma taipoxin-2 precursor [Oxyuranus scutellatus scutellatus]	Oxyuranus scutellatus scutellatus
15	0,00	4,00	14,29	gi 129446	RecName: Full=Phospholipase A2, taipoxin gamma chain; AltName: Full=Phosphatidylcholine 2-acylhydrolase	Oxyuranus scutellatus scutellatus
16	2,00	2,00	3,27	gi 145982762	scutellatease-1 [Oxyuranus scutellatus]	Oxyuranus scutellatus

Table 1, Escoffier et al.

Figure 1

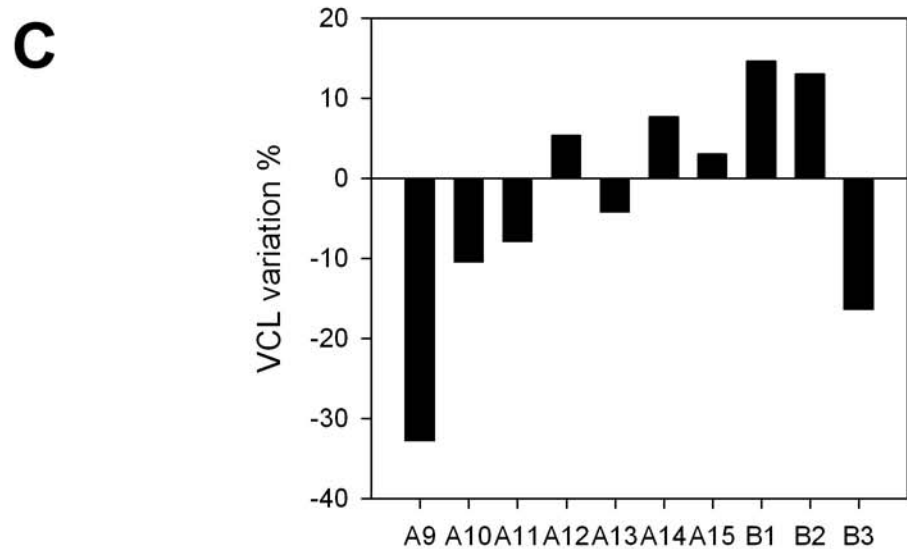
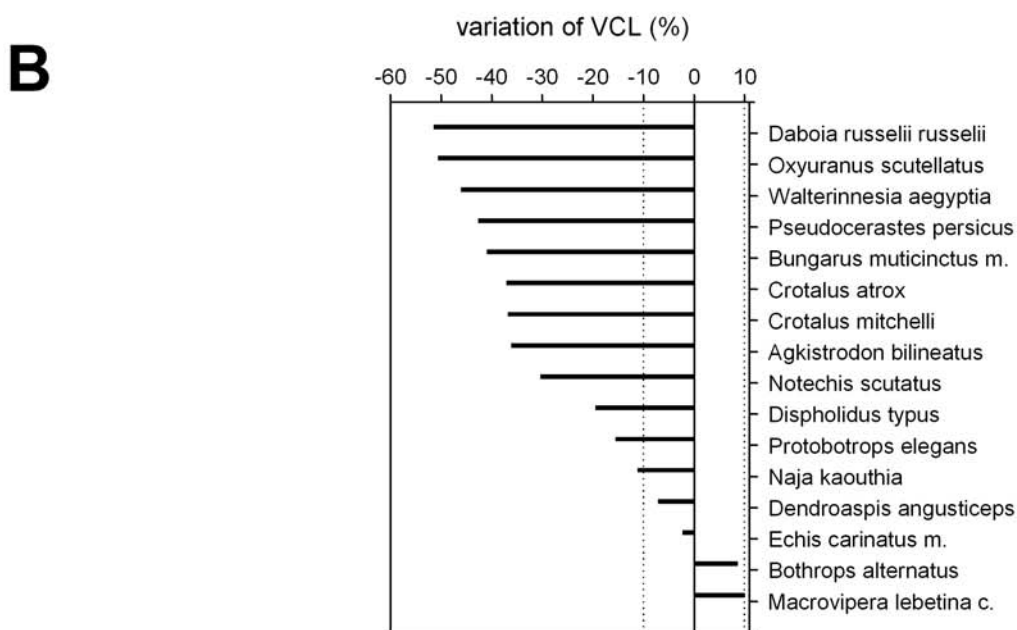
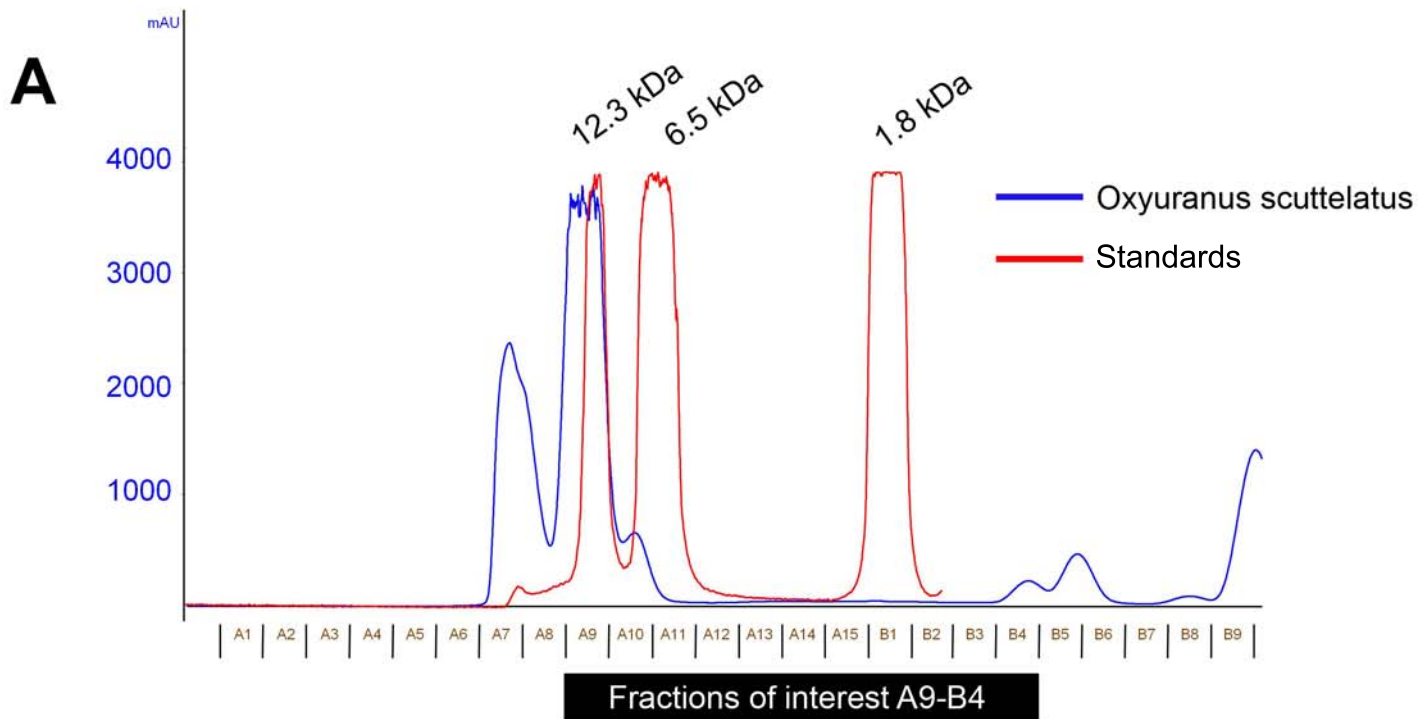
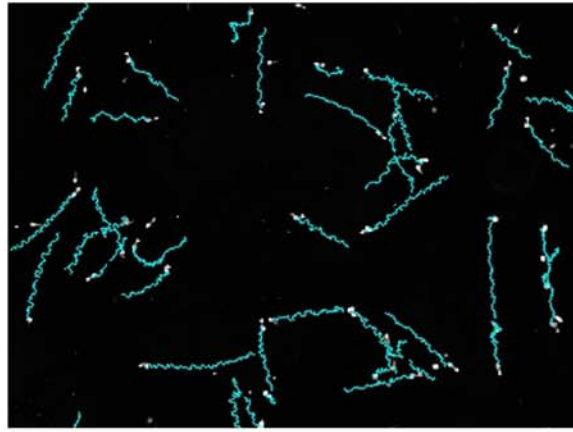


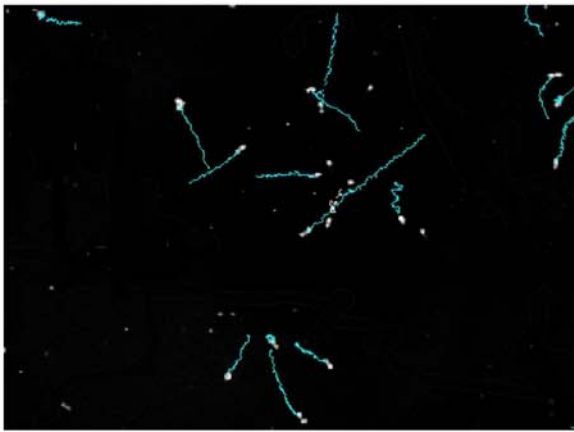
Figure 1, Escoffier et al

Figure 2

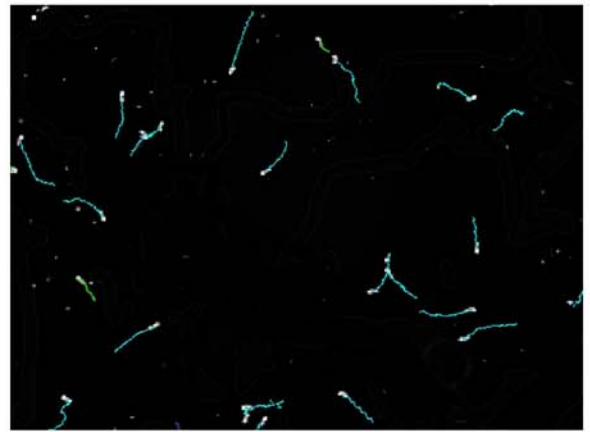
A



Control

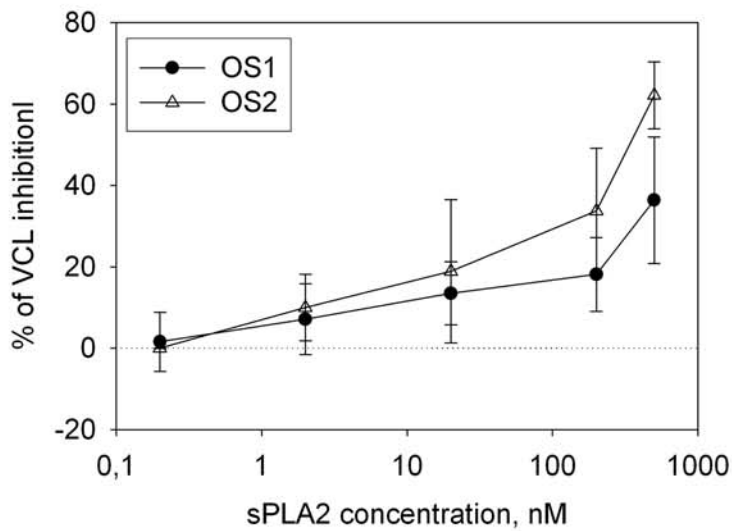


+ 200 nM OS1



+ 200 nM OS2

B



C

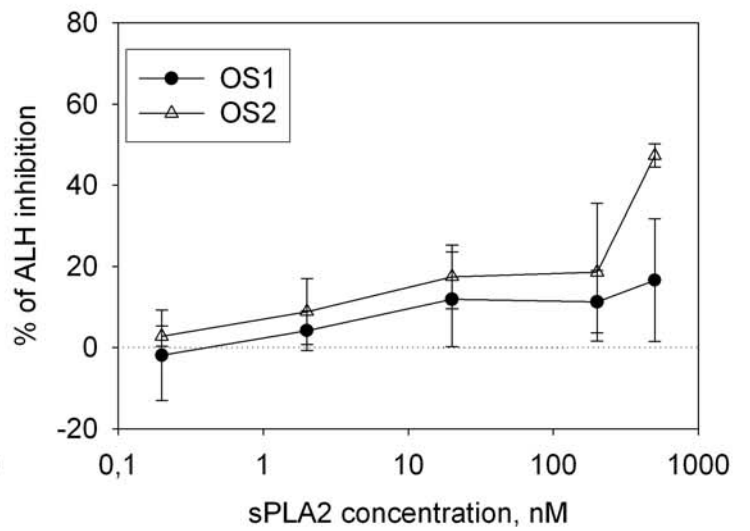


Figure 2, Escoffier et al.

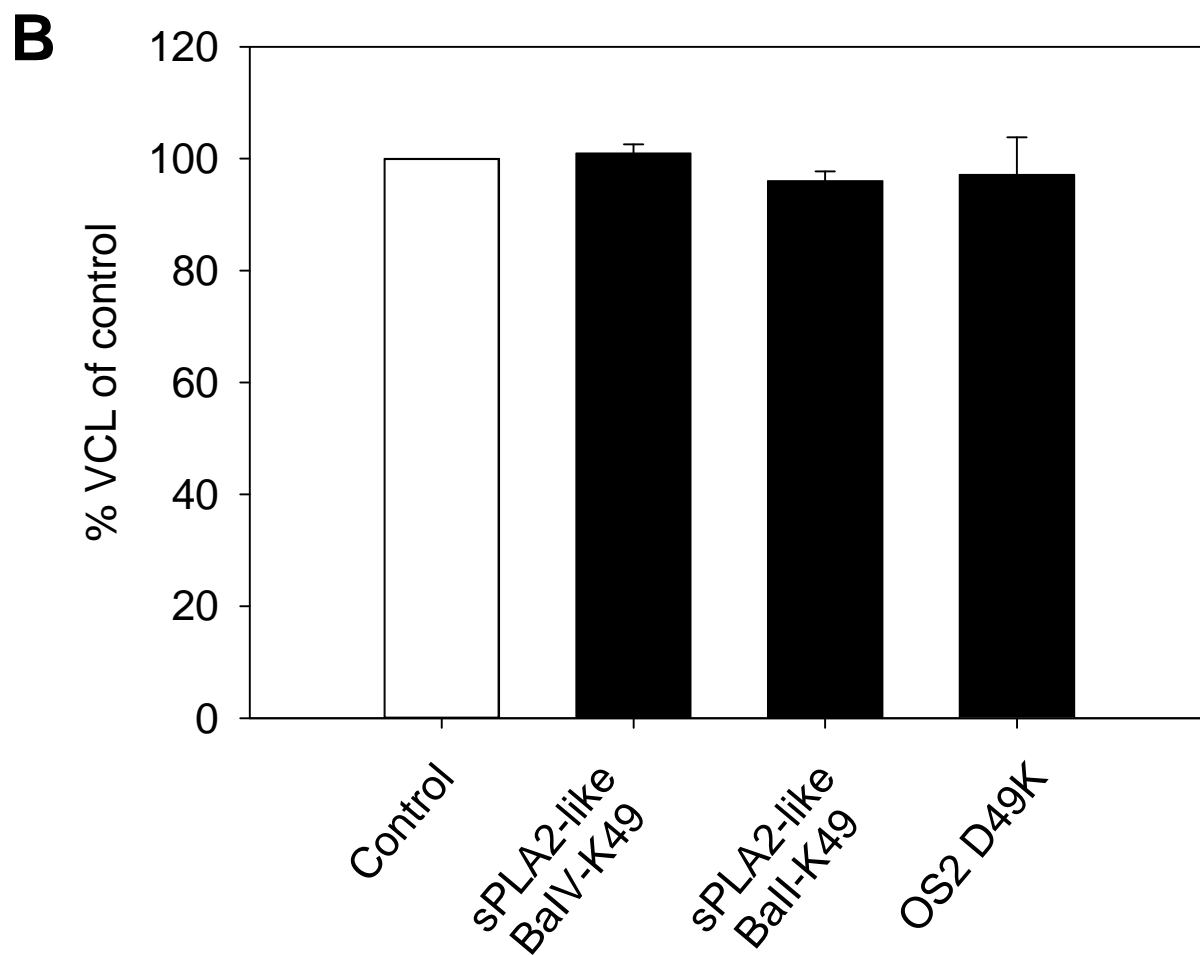
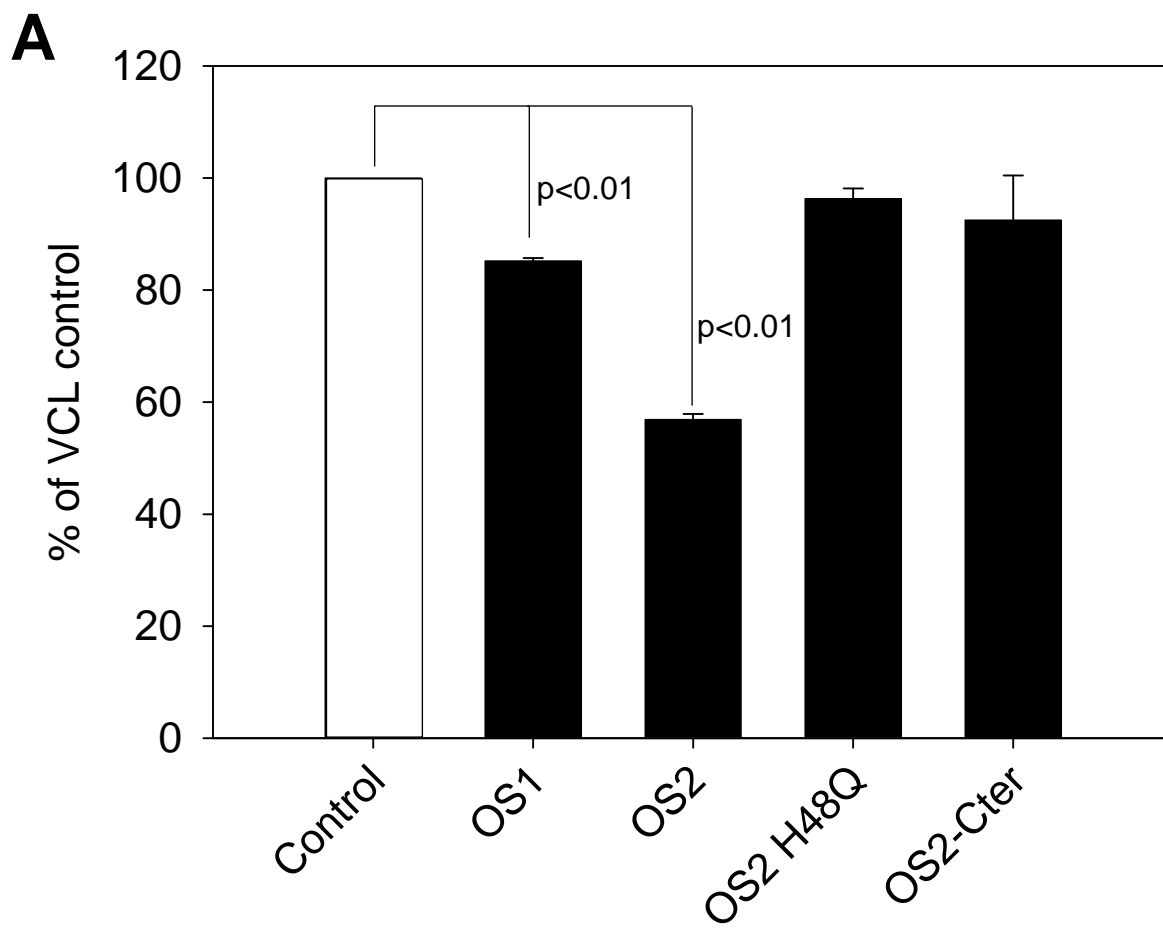
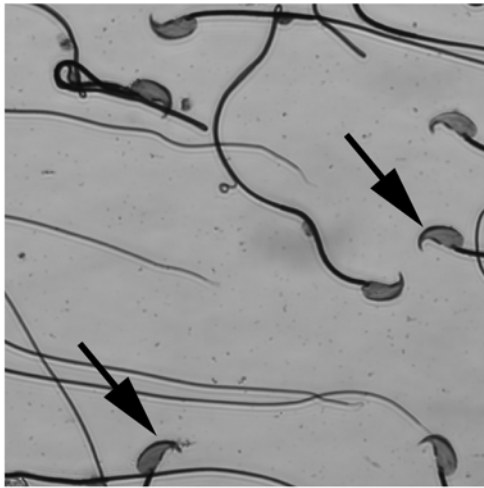


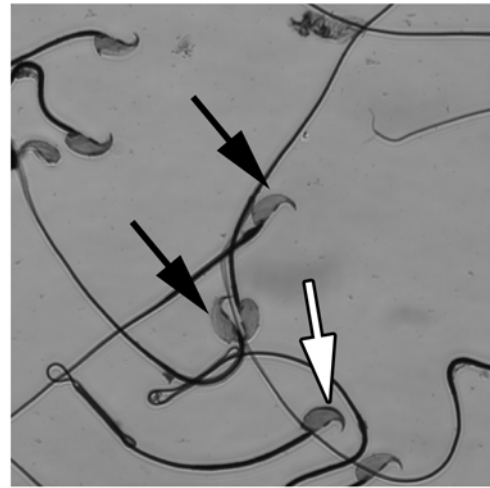
Figure 4

A



Control

B



+ OS2

C

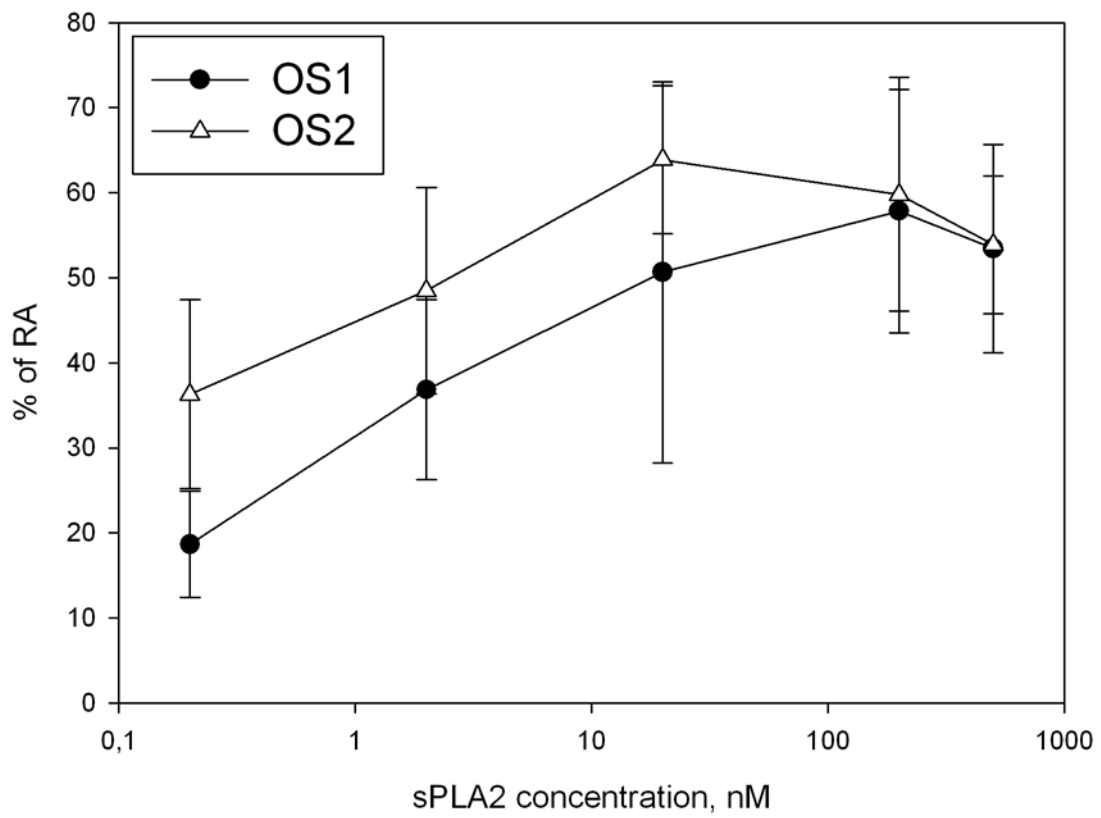
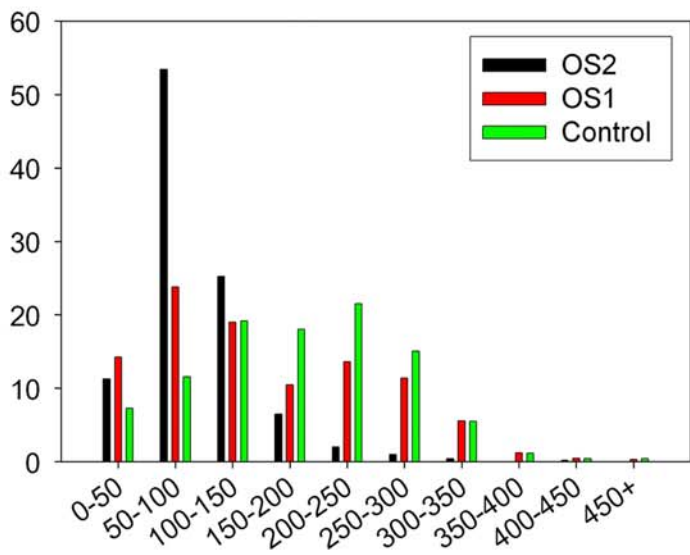


Figure 4, Escoffier et al.

Figure 5

A



B

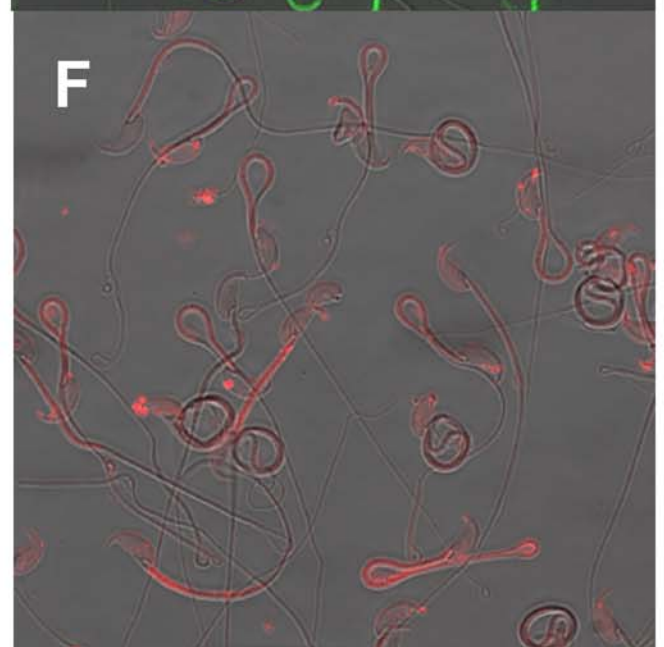
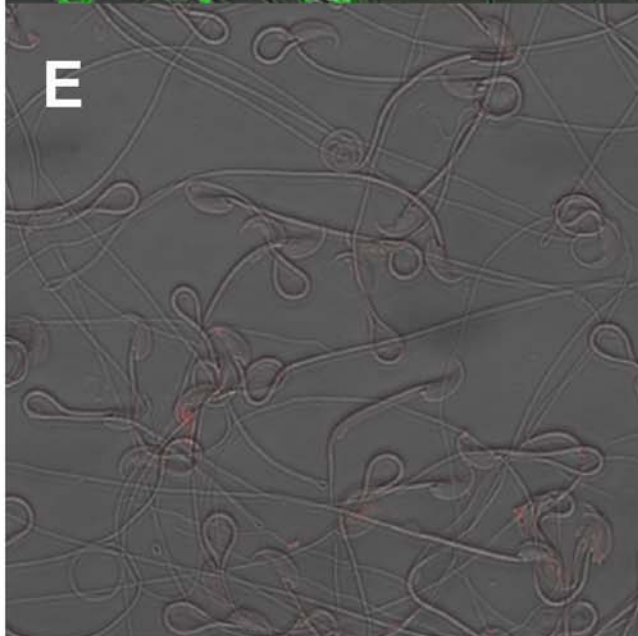
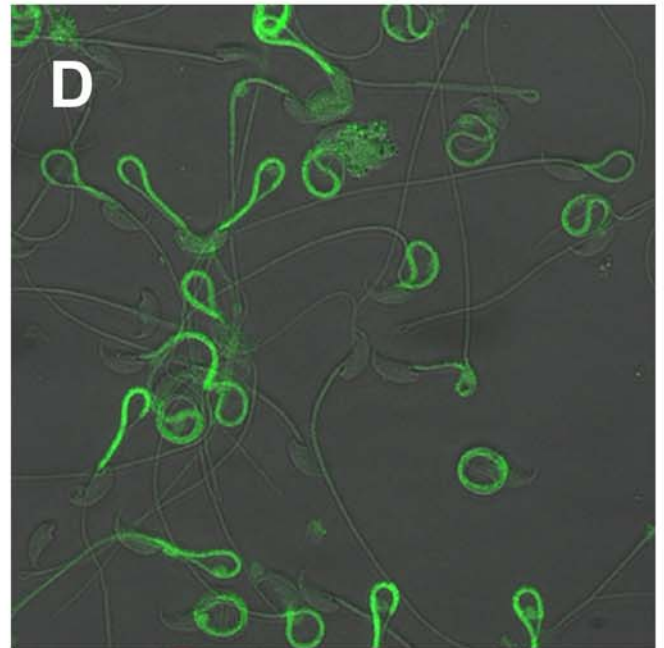
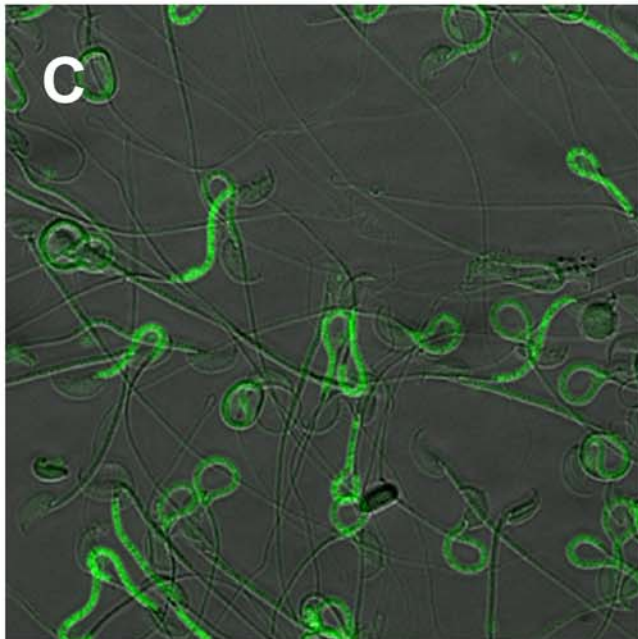
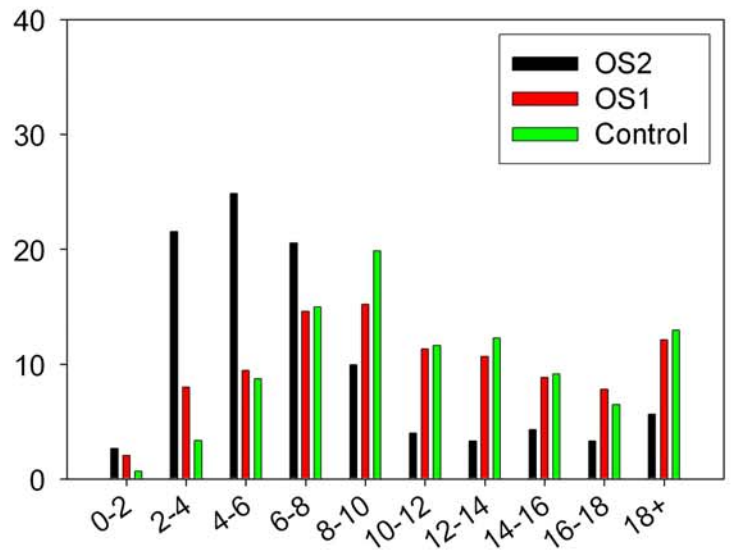
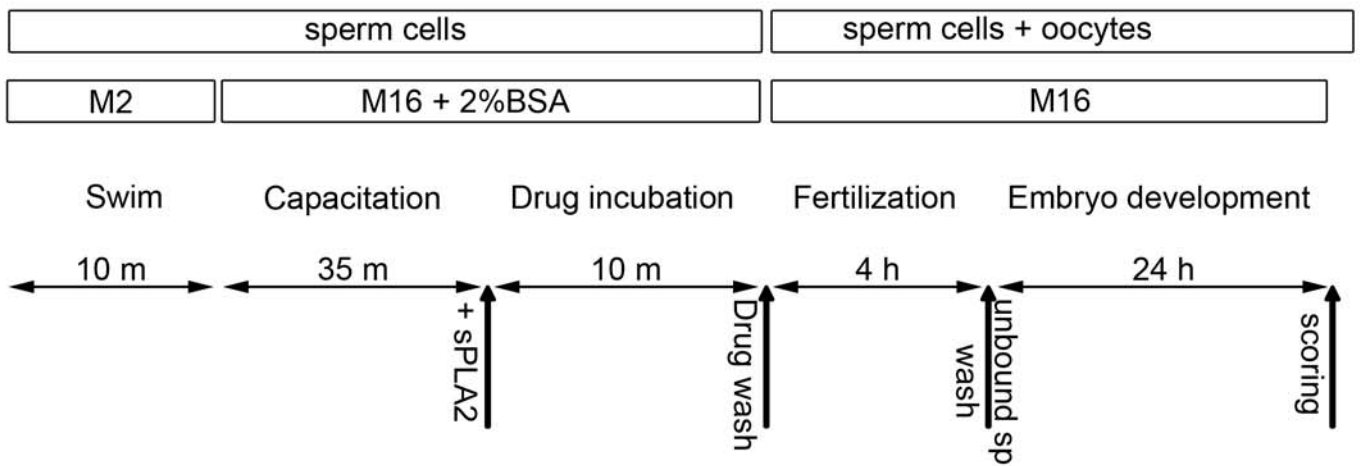
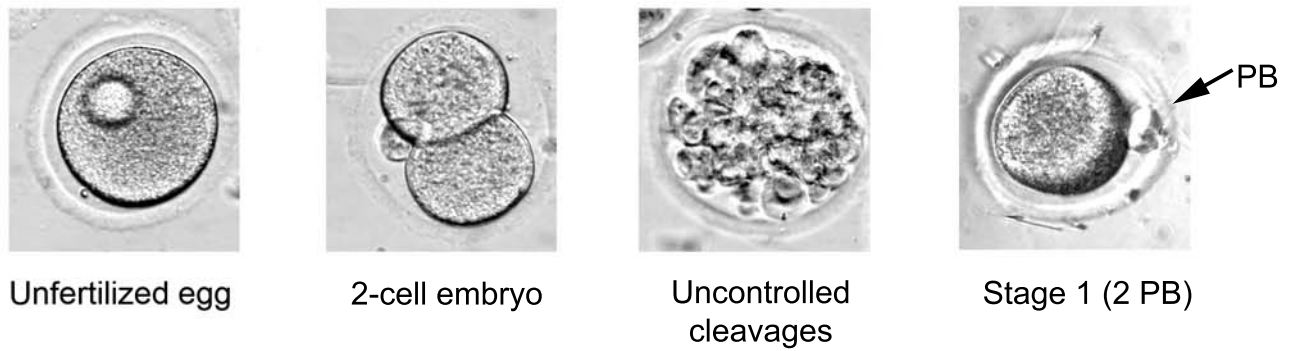


Figure 5, Escoffier et al.

A



B



C

