

## Supplementary Material

### **Generation of reproductively mature offspring from the endangered green and golden bell frog *Litoria aurea* using cryopreserved spermatozoa**

*Rose Upton*<sup>A,B,G</sup>, *Simon Chulow*<sup>B,C</sup>, *Natalie E. Calatayud*<sup>B,D,E</sup>, *Kim Colyvas*<sup>F</sup>, *Rebecca G. Y. Seeto*<sup>A</sup>,  
*Lesley A. M. Wong*<sup>A</sup>, *Michael J. Mahony*<sup>A,B</sup> and *John Chulow*<sup>A,B</sup>

<sup>A</sup>The Conservation Biology Research Group, School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW 2308, Australia.

<sup>B</sup>FAUNA Research Alliance, PO Box 5092, Kahibah, NSW 2290, Australia.

<sup>C</sup>Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia.

<sup>D</sup>Taronga Institute of Science and Learning, Taronga Conservation Society Australia, Taronga Western Plains Zoo, Dubbo, NSW 2830, Australia.

<sup>E</sup>San Diego Zoo Global-Beckman Center for Conservation Research, 15600 San Pasqual Valley Road, Escondido, CA 92027, USA.

<sup>F</sup>School of Mathematical and Physical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

<sup>G</sup>Corresponding author. Email: [rose.upton@uon.edu.au](mailto:rose.upton@uon.edu.au)

**Table S1. Sperm recovery, Model 1 (treatments only, no controls)**

Summary of results comparing effectiveness of DMSO and glycerol at concentrations of 1.4, 2.1 and 2.8 M for forward-progressive and non-progressive motility and vitality (membrane intactness). OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit. Confidence limits = 95%

Category	Contrasts	OR	LCL	UCL
Forward	DMSO/Glycerol	6.7	3.7	12.0
progressive	1.4/2.1	1.4	0.7	2.8
Motility	1.4/2.8	4.0	2.0	8.2
	2.1/2.8	2.9	1.4	5.9
Non-	DMSO/Glycerol	3.2	2.0	5.0
progressive	1.4/2.1	0.9	0.5	1.6
Motility	1.4/2.8	1.1	0.6	1.8
	2.1/2.8	1.2	0.7	2.1
Vitality	DMSO/Glycerol	2.0	1.4	3.0
	1.4/2.1	0.8	0.5	1.3
	1.4/2.8	0.8	0.5	1.3
	2.1/2.8	1.0	0.6	1.6
Acrosome Integrity	DMSO/Glycerol	1.2	1.0	1.5
	1.4/2.1	1.2	0.9	1.6
	1.4/2.8	1.4	1.1	1.8
	2.1/2.8	1.2	0.9	1.4

**Table S2. Sperm recovery, Model 2 (treatments and controls)**

Odds Ratios and Confidence Intervals for Forward-progressive motility, non-progressive motility and vitality of cryopreserved sperm. OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; 1.4d = 1.4 M DMSO; 2.1d = 2.1 M DMSO; 2.8d = 2.8 M DMSO; 1.4g = 1.4 M glycerol; 2.1g = 2.1 M glycerol; 2.8g = 2.8 M glycerol; 0.3s = 0.3 M sucrose

Contrast	Forward-progressive Motility			Non-progressive Motility			Vitality		
	OR	LCL	UCL	OR	LCL	UCL	OR	LCL	UCL
uf/1.4d	12.2	4.7	31.5	5.6	2.7	11.9	60.9	24.7	150.3
uf/2.1d	8.7	3.4	22.3	5.5	2.6	11.6	60.4	24.5	148.7
uf/2.8d	25.8	9.8	67.7	3.7	1.7	7.7	51.2	20.8	126.3
uf/1.4g	33.2	12.7	86.7	13.2	6.2	28.1	145.6	59.0	359.3
uf/2.1g	96.3	35.0	264.4	10.6	5.0	22.4	98.2	39.8	242.4
uf/2.8g	263.9	94.6	736.0	22.4	10.8	46.7	108.5	44.8	263.0
uf/0.3s	53.5	20.2	141.9	25.5	11.8	54.8	503.6	201.0	1261.6
1.4d/2.8d	2.1	0.9	5.1	0.7	0.3	1.3	0.8	0.4	1.6
1.4d/1.4g	2.7	1.1	6.5	2.4	1.2	4.6	2.4	1.2	4.6
1.4d/2.1g	7.9	3.1	20.0	1.9	1.0	3.7	1.6	0.8	3.1
1.4d/2.8g	21.7	8.4	55.8	4.0	2.1	7.6	1.8	0.9	3.4
1.4d/0.3s	4.4	1.8	10.7	4.6	2.3	9.0	8.3	4.2	16.3
2.1d/1.4d	1.4	0.6	3.3	1.0	0.5	2.0	1.0	0.5	1.9
2.1d/2.8d	3.0	1.2	7.2	0.7	0.4	1.3	0.8	0.4	1.6
2.1d/1.4g	3.8	1.6	9.2	2.4	1.2	4.7	2.4	1.3	4.6
2.1d/2.1g	11.1	4.4	28.0	1.9	1.0	3.7	1.6	0.8	3.1
2.1d/2.8g	30.5	11.9	78.2	4.1	2.2	7.8	1.8	1.0	3.4
2.1d/0.3s	6.2	2.5	15.0	4.7	2.4	9.2	8.3	4.2	16.4
2.8d/1.4g	1.3	0.5	3.2	3.6	1.9	7.0	2.8	1.5	5.5
2.8d/2.1g	3.7	1.5	9.6	2.9	1.5	5.6	1.9	1.0	3.7
2.8d/2.8g	10.2	3.9	26.8	6.2	3.3	11.6	2.1	1.1	4.0
2.8d/0.3s	2.1	0.1	0.5	7.0	3.6	13.6	9.8	5.0	19.3
1.4g/2.1g	2.9	1.1	7.4	0.8	0.4	1.6	0.7	0.3	1.3
1.4g/2.8g	8.0	3.1	20.7	1.7	0.9	3.3	0.7	0.4	1.4

1.4g/0.3s	1.6	0.7	4.0	1.9	1.0	3.8	3.5	1.8	6.8
2.1g/2.8g	2.7	1.0	7.5	2.1	1.1	4.0	1.1	0.6	2.1
0.3s/2.1g	1.8	0.7	4.7	0.4	0.2	0.8	0.2	0.1	0.4
0.3s/2.8g	4.9	1.9	13.0	0.9	0.5	1.7	0.2	0.1	0.4

---

**Table S3. Sperm recovery, Model 2 (treatments and 0.3 M sucrose control)**

Odds Ratios and Confidence Intervals for intact acrosomes. OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; 1.4d = 1.4 M DMSO; 2.1d = 2.1 M DMSO; 2.8d = 2.8 M DMSO; 1.4g = 1.4 M glycerol; 2.1g = 2.1 M glycerol; 2.8g = 2.8 M glycerol; 0.3s = 0.3 M sucrose

Contrast	OR	LCL	UCL
1.4d/2.8d	1.4	1.0	1.9
1.4d/1.4g	1.2	0.9	1.8
1.4d/2.1g	1.4	1.0	2.0
1.4d/2.8g	1.7	1.2	2.4
1.4d/0.3s	1.7	1.2	2.5
2.1d/1.4d	0.8	0.6	1.1
2.1d/2.8d	1.1	0.8	1.5
2.1d/1.4g	1.0	0.7	1.4
2.1d/2.1g	1.1	0.8	1.6
2.1d/2.8g	1.4	1.0	1.9
2.1d/0.3s	1.4	0.9	2.0
2.8d/1.4g	0.9	0.6	1.2
2.8d/2.1g	1.1	0.8	1.5
2.8d/2.8g	1.3	0.9	1.7
2.8d/0.3s	1.2	0.9	1.8
1.4g/2.1g	1.2	0.8	1.7
1.4g/2.8g	1.4	1.0	1.9
1.4g/0.3s	1.4	0.9	2.0
2.1g/2.8g	1.2	0.9	1.7
0.3s/2.1g	0.8	0.6	1.2
0.3s/2.8g	1.0	0.7	1.4

**Table S4. Odds Ratios and 95% Confidence Intervals for survival of embryos to first cleavage**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	3.1	0.8	12.4
uf/cr-d	42.3	10.3	174.3
uf/cr-g	62.6	14.9	263.4
uf/uf-g	47.3	11.3	198.3
uf-d/cr-d	13.7	3.4	55.6
uf-d/cr-g	20.2	4.9	84.1
uf-d/uf-g	15.3	3.7	63.3
cr-d/cr-g	1.5	0.4	6.3
cr-d/uf-g	1.1	0.3	4.7
cr-g/uf-g	0.8	0.2	3.2

**Table S5. Odds Ratios and 95% Confidence Intervals for survival of embryos to second cleavage**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	2.9	0.5	16.0
uf/cr-d	44.0	7.6	253.4
uf/cr-g	183.2	27.4	1224.2
uf/uf-g	62.6	10.3	382.3
uf-d/cr-d	15.4	2.7	88.5
uf-d/cr-g	64.2	9.6	429.0
uf-d/uf-g	21.9	3.6	134.1
cr-d/cr-g	4.2	0.6	28.0
cr-d/uf-g	1.4	0.2	8.7
cr-g/uf-g	0.3	0.1	2.4

**Table S6. Odds Ratios and 95% Confidence Intervals for survival to multicellular embryos**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	2.9	0.5	17.3
uf/cr-d	55.8	9.1	342.46
uf/cr-g	500.0	56.9	4393.36
uf/uf-g	63.7	10.0	407.7
uf-d/cr-d	19.0	3.1	116.6
uf-d/cr-g	170.6	19.3	1505.2
uf-d/uf-g	21.7	3.4	139.4
cr-d/cr-g	9.0	1.0	79.6
cr-d/uf-g	1.1	0.2	7.4
cr-g/uf-g	0.1	0.01	1.2



**Table S7. Odds Ratios and 95% Confidence Intervals for survival of embryos to blastulation**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	3.2	0.6	16.0
uf/cr-d	48.8	9.3	255.2
uf/cr-g	416.4	55.4	3129.8
uf/uf-g	36.9	7.1	190.3
uf-d/cr-d	15.2	2.9	79.7
uf-d/cr-g	130.1	17.2	982.2
uf-d/uf-g	11.5	2.2	59.4
cr-d/cr-g	8.5	1.1	65.3
cr-d/uf-g	0.8	0.1	4.0
cr-g/uf-g	0.1	0.01	0.7

**Table S8. Odds Ratios and 95% Confidence Intervals for survival of embryos to gastrulation**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	3.0	1.0	11.0
uf/cr-d	50.0	13.4	188.0
uf/cr-g <sup>a</sup>	6.0x10 <sup>9</sup>	0.0	Inf
uf/uf-g	58.0	14.9	223.0
uf-d/cr-d	16.0	4.2	59.0
uf-d/cr-g <sup>A</sup>	1.9x10 <sup>9</sup>	0.0	Inf
uf-d/uf-g	18.0	4.7	69.0
cr-d/cr-g <sup>A</sup>	1.2x10 <sup>8</sup>	0.0	Inf
cr-d/uf-g	1.0	0.3	5.0
cr-g/uf-g	0.0	0.0	Inf

<sup>A</sup>Extreme values for the OR's and CI's are due to numerical problems in the estimation due to zero occurrences for condition(s) involved in the ratios.

**Table S9. Odds Ratios and 95% Confidence Intervals for survival of embryos to neurulation**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	5.0	0.9	23.0
uf/cr-d	52.0	9.1	300.0
uf/cr-g <sup>A</sup>	2.2x10 <sup>10</sup>	0.0	9.3x10 <sup>285</sup>
uf/uf-g	188.0	22.1	1600.0
uf-d/cr-d	11.0	2.0	650.0
uf-d/cr-g <sup>A</sup>	4.69x10 <sup>9</sup>	0.0	2.0x10 <sup>285</sup>
uf-d/uf-g	41.0	4.9	340.0
cr-d/cr-g <sup>A</sup>	4.1x10 <sup>8</sup>	0.0	1.8x10 <sup>284</sup>
cr-d/uf-g	4.0	0.4	320.0
cr-g/uf-g <sup>A</sup>	0.0	0.0	3.8x10 <sup>267</sup>

<sup>A</sup>Extreme values for the OR's and CI's are due to numerical problems in the estimation due to zero occurrences for condition(s) involved in the ratios.

**Table S10. Odds Ratios and 95% Confidence Intervals for survival of embryos to organogenesis**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	5.0	1.1	21.0
uf/cr-d	61.0	11.4	321.0
uf/cr-g <sup>A</sup>	1.8x10 <sup>10</sup>	0.0	Inf
uf/f-g	169.0	22.4	1281.0
uf-d/cr-d	13.0	2.4	68.0
uf-d/cr-g <sup>A</sup>	3.7x10 <sup>9</sup>	0.0	Inf
uf-d/uf-g	35.0	4.8	262.0
cr-d/cr-g <sup>A</sup>	2.9x10 <sup>8</sup>	0.0	Inf
cr-d/uf-g	3.0	0.3	23.0
cr-g/uf-g <sup>A</sup>	0.0	0.0	Inf

<sup>A</sup>Extreme values for the OR's and CI's are due to numerical problems in the estimation due to zero occurrences for condition(s) involved in the ratios.

**Table S11. Odds Ratios and 95% Confidence Intervals for survival of embryos to hatching tadpoles**

OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; uf = unfrozen sperm; uf-d = unfrozen 2.1 M DMSO sperm; cr-d = cryopreserved 2.1 M DMSO sperm; uf-g = unfrozen 2.1 M glycerol sperm; cr-g = cryopreserved 2.1 M glycerol sperm

Contrast	OR	LCL	UCL
uf/uf-d	2.0	0.5	6.0
uf/cr-d	34.0	7.2	164.0
uf/cr-g <sup>A</sup>	3.7x10 <sup>9</sup>	0.0	Inf
uf/uf-g	62.0	10.6	364.0
uf-d/cr-d	19.0	4.0	93.0
uf-d/cr-g <sup>A</sup>	2.1x10 <sup>9</sup>	0	Inf
uf-d/uf-g	35.	5.9	206.0
cr-d/cr-g <sup>A</sup>	1.1x10 <sup>8</sup>	0	Inf
cr-d/uf-g	2.0	0.3	12.0
cr-g/uf-g	0.0	0.0	Inf

<sup>A</sup>Extreme values for the OR's and CI's are due to numerical problems in the estimation due to zero occurrences for condition(s) involved in the ratios.