In vitro and *in vivo* survival of mouse morulas and blastocysts following vitrification in 45% glycerol

Sobrevivência *in vitro* e *in vivo* de mórulas e blastocistos murinos vitrificados em meio contendo 45% de glicerol

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ABSTRACT

The toxicity of cryoprotectant agents is one of the critical factors for the successful vitrification of mammalian embryos, which depends on the concentration, time and temperature of exposure to the cryoprotectant. Moreover, embryos from different species or stages of development have distinct levels of tolerance to cryoprotectant agents. This study aimed to evaluate the *in vitro* and *in vivo* survivals of mouse embryos after distinct times of exposure to two cryoprotectant concentrations prior to vitrification. In Experiment 1, compact morulas, blastocysts and expanded blastocysts were exposed to 10% glycerol for 10 min (group 1) or to 25% glycerol for 10, 5 and 2.5 min (groups 2, 3, and 4, respectively) prior to their exposure to the vitrification solution containing 45% glycerol for 1 min at 20°C before immersion in liquid nitrogen. Embryos were thawed in a water bath at 20°C for 20 sec, and the cryoprotectant was diluted in 1 M sucrose for 10 min. Then, embryos were morphologically evaluated following *in vitro* culture for 1 and 48 h. *In vitro* survivals of compact morulas and blastocysts were not affected by glycerol concentration and/or equilibration time prior to vitrification. However, expanded blastocysts demonstrated a lower survivability to vitrification. In Experiment 2, fresh and vitrified (according to procedures in group 1, Experiment 1) compact morulas and blastocysts were transferred to recipients following *in vitro* culture for 1 h at room temperature. Pregnancy rates, based on the proportion of viable fetuses, were similar between vitrified and fresh compact morulas (27% and 33%, respectively). However, vitrified blastocysts demonstrated a lower *in vivo* survival than controls (9% vs. 52%, respectively).

Key words: vitrification, cryopreservation, embryo, mouse.

RESUMO

A toxidez do agente crioprotetor é um dos aspectos mais críticos no sucesso da vitrificação de embriões mamíferos, variando conforme a concentração, o tempo e a temperatura de exposição. Além disto, embriões de diferentes espécies ou estádios de desenvolvimento podem tolerar diferentes níveis de exposição a agentes crioprotetores. Este estudo visou avaliar a sobrevivência *in vitro* e *in vivo* de embriões *Mus musculus* após a vitrificação em diferentes concentrações e tempos de exposição ao crioprotetor previamente à vitrificação. No Experimento 1, mórulas compactas, blastocistos e blastocistos expandidos foram expostos a 10% de glicerol por 10 min (grupo 1) ou 25% de glicerol por 10, 5 ou 2,5 min (grupos 2, 3 e 4) previamente à exposição à solução de vitrificação contendo 45% de glicerol por 1 min, a 20°C, antes da imersão em nitrogênio líquido. A descongelação ocorreu em banho-maria a 20°C por 20 seg, e a diluição do crioprotetor foi realizada em 1 M de sacarose por 10 min. Após cultivo *in vitro* por 1 e 48 h, os embriões foram avaliados morfologicamente. A sobrevivência *in vitro* de mórulas compactas e blastocistos não foi afetada pelas concentrações e tempos de equilíbrio em glicerol previamente à vitrificação. No entanto, houve uma menor viabilidade embrionária após a vitrificação de blastocistos expandidos. No Experimento 2, mórulas compactas e blastocistos frescos e vitrificados (conforme o grupo 1, do Experimento 1) foram transferidos para receptoras após cultivo *in vitro* de 1 h. As taxas de prenhez, baseadas na proporção de fetos viáveis, foram semelhantes entre mórulas compactas vitrificadas e frescas (27 vs. 33%, respectivamente). Entretanto, os blastocistos vitrificados mostraram uma sobrevivência *in vivo* menor que os controles (9% vs. 52%, respectivamente).

Descritores: vitrificação, criopreservação, embrião, camundongo.

INTRODUCTION

Since the successful cryopreservation of the mouse embryo [30,32], many freezing methods have been devised in a number of species in an attempt to promote cell survival and reduce ice crystal formation, osmotic stress, and cell damage during the freezing process. Simply stated, freezing is the solidification of a liquid [13], which can occur either by crystallization, involving an ordered molecule arrangement, or by vitrification, by the extreme elevation in viscosity during cooling, with no ice crystal formation [8,12].

Due to its simplicity and relative success in embryo freezing, vitrification has become a theme of intense investigation in the past few years. During vitrification, cells are pre-dehydrated prior to cooling by their exposure to concentrated cryoprotectant solutions. Consequently, potential injuries due to the lack of ice crystal formation during freezing are minimized [12,20]. However, cryoprotectant toxicity and osmotic injuries to embryos still may occur during vitrification. In this regard, the use of cryoprotectants of lower toxicity, and the determination of optimal cryoprotectant concentrations, and time, volume and temperature of exposure are strategies commonly used to minimize harmful effects to cells [20].

Various other aspects related to embryo freezing also depend on poorly understood biological factors, such as species, stage of development, and cell properties (e.g., cell surface: volume ratio, membrane permeability, lipid composition). The aim of this study was to determine the *in vitro* and *in vivo* survival of mouse embryos at distinct stages of development after pre-dehydration with different glycerol concentrations and exposure times prior to vitrification in 45% glycerol.

MATERIALS AND METHODS

Production of mouse embryos

Six- to 8-weeks old Swiss CF1 mouse females on a 14 h light/10 h dark cycle were superovulated with the intraperitonial injection of 10 IU eCG (Folligon[1]) and 10 IU hCG (Pregnyl[2]), 46 to 48 h apart. Following the hCG injection, donors females were mated overnight with fertile males (1 to 3 donors/male), whereas recipient females were induced were mated with vasectomized males to induce pseudopregnancy. The presence of a vaginal plug was checked early next morning (Day 1), and positive donor females were separated and sacrificed on Day 4 (90 to 92 h after the hCG injection)

by cervical dislocation. Uterine horns were individually flushed with 0.5 mL holding medium, composed of Dulbecco's PBS supplemented with 20% heat-inactivated castrated steer serum (CSS). Structures obtained upon collection were washed and selected under a stereomicroscope. Embryos were morphologically evaluated based on developmental stage and quality (grades 1, 2, 3, and 4), according to procedures described elsewhere [21]. Grade 1 (excellent) compact morulas, blastocysts and expanded blastocysts were randomly allocated to the experimental groups.

Embryo vitrification and thawing

Two experiments were carried out for the evaluation of the *in vitro* and *in vivo* survival of mouse embryos after vitrification using glycerol as the cryoprotectant agent. In Experiment 1, compact morulas (n=173), blastocysts (n=173) and expanded blastocysts (n=93) were equilibrated at RT for pre-dehydration using intracellular solutions at two distinct concentrations (10 or 25% glycerol in holding medium) and three exposure times (2.5, 5, or 10 min), arranged into four distinct groups, as follows: (1) 10% glycerol for 10 min; (2) 25% glycerol for 10 min; (3) 25% glycerol for 5 min; and (4) 25% glycerol for 2.5 min. Following equilibration, groups of 5 to 20 embryos were pipetted directly into 0.5 mL straws containing 0.15 mL vitrification solution (45% glycerol in holding medium). After exposure for 1 min at 20°C, straws were plunged into LN_2 and properly stored. In Experiment II, compact morulas (n=249) and blastocysts (n=227) were vitrified following procedures used in Group 1 in Experiment 1: equilibrium for 10 min in 10% glycerol, exposure for 1 min to 45% glycerol, followed by immersion in LN_2.

Embryos from both experiments were thawed by the exposure of each straw to air for 5 to 8 sec, followed by their immersion in a water bath at 20°C for 20 sec. The cryoprotectant removal was carried out using a 1 M sucrose solution for 10 min at RT. Frozen-thawed embryos were rinsed in holding medium and *in vitro* cultured for 60 min at 37°C and 95% humidity before morphological evaluation.

Experiment 1: *In vitro* embryo survival

Viable embryos (grades 1, 2, and 3) at 60 min post-thawing were *in vitro* cultured in 100 µl drops of Whitten medium + 20% CSS, under oil, for 48 h, at 37°C, 95% humidity and 5% CO_2 in air. Embryo survival was assessed by their ability to develop to the ex-

panded and hatched blastocyst stages following *in vitro* culture.

Experiment 2: *In vivo* embryo development

Grades 1 and 2 compact morulas and blastocysts at 60 min post-thawing were surgically transferred to the right uterine horns (5 to 10 embryos per horn) of pseudopregnant recipients on Day 3 (-1 day relative to donors). Fresh embryos (control group) were transferred to the left uterine horn of each recipient female.

A total of 358 vitrified (184 compact morulas and 174 blastocysts) and 401 fresh embryos were transferred to 49 recipients. Recipients were subjected to euthanasia on Day 15 of pregnancy to determine pregnancy rates and the total number of implantation sites and developing fetuses. The relative efficiency (expressed in %) was calculated as the ratio between the percentage of implantations or fetuses obtained into each treatment group by the percentage obtained in the respective control groups.

Statistical Analysis

Data regarding *in vitro* and *in vivo* embryo survival were analyzed by the χ^2 or by the Fisher's tests, depending on the sample numbers, for a level of significance of 5% (P<0.05).

RESULTS

Experiment 1: *In vitro* survival of embryos

The results of embryo viability observed 1 h and 48 h after thawing and *in vitro* culture (IVC) are summarized in Table 1. The mean recovery rate after thawing was 89% (432/487), and it was not different among stages of development or treatment groups.

Significant differences existed in embryo survival and development between stages of development and treatment groups at 1 and 48 h post-thawing. In general, *in vitro* survival was not affected by the glycerol concentration (10 or 25%) or by the equilibration time (2.5, 5, or 10 min) prior to vitrification. However, blastocysts and expanded blastocysts demonstrated a lower survivability to vitrification. For vitrified compact morulas, *in vitro* survival at 1h post-thawing was similar among treatments, but the exposure for 10 min in 10% or 25% glycerol (Groups 1 or 2) prior to vitrification promoted higher rates of development to the expanded or hatched blastocyst stages after 48 hours of IVC than the exposure for 2.5 min at 25% glycerol (Group 4).

Vitrified blastocysts had similar *in vitro* development among treatments following short (1 h) and long term (48 h) IVC. This was also true for vitrified expanded blastocysts 48 h after thawing, but not at 1h. However, *in vitro* survival rates for expanded blastocysts were generally lower than compact morulas and blastocysts at 48 h post-thawing, which were generally similar to one another between groups.

Experiment 2: *In vivo* development of embryos

Table 2 shows the occurrence of implantation sites and viable fetuses after the transfer of fresh and vitrified morulas and blastocysts to female recipients. Pregnancy rate for female recipients having at least one implantation site on Day 15 of development was 31% (15/49). Pregnancy rates based on the number of embryos transferred per pregnant female were not different between fresh and vitrified embryos (124/401 or 31% and 197/358 or 30%, respectively), irrespective of the stage of development or the vitrification treatment. In addition, no differences were observed in number of implantations and fetuses after the transfer of fresh or vitrified compact morulas. However, a significant difference in development existed considering the number of implantation sites and viable fetuses between fresh and vitrified blastocysts, with a decrease of approximately 80% in viability following the transfer of vitrified blastocysts compared with the control counterparts.

The relative efficacies of the vitrification of compact morulas and blastocysts in 45% glycerol were 98% and 24% for implantations, respectively, and 82% and 17% for fetuses, respectively, relative to controls.

DISCUSSION

There are several methods available to estimate embryo survival following freezing, but the morphological evaluation of embryos after thawing is still considered one of the most useful and practical approaches. When performed after a short-term *in vitro* culture, to allow the embryo to recover from freezing injuries, or for the detection of cell damage that otherwise would not be visible soon after thawing [25-27], the morphological evaluation is highly correlated with *in vivo* development following transfer to surrogate females in mice [28,29]. Our results indicated that the evaluation of vitrified compact morulas 1 h post-thawing was accompanied by a high rate of *in vitro* survival after IVC and a similar rate of *in vivo* development as observed for

Table 1. *In vitro* survival of mouse embryos vitrified at distinct stages of development after exposure to different glycerol concentrations and equilibration times.

Stage of development	Experimental group[1]	Recovered embryos after thawing[2]		Embryo survival after 1		Developmental rate after 48 h IVC[3]	
		N	%	N	%	N	%
Compact morula	1	48/54	89	46[b]	96	28[a]	58
	2	41/47	87	35[a,b,c,d]	85	27[a]	66
	3	39/52	75	33[a,b,c,d]	85	20[a,b,c]	51
	4	45/46	98	43[b]	96	15[b,c,d]	33
Blastocyst	1	52/60	87	47[a,b,d]	90	33[a]	63
	2	40/42	95	32[a,c,d]	80	21[a,b]	53
	3	40/44	91	38[b,d]	95	22[a,b]	55
	4	41/46	89	35[a,b,c,d]	85	19[a,b,c,d]	46
Expanded blastocyst	1	21/22	95	15[a]	71	4[d]	19
	2	22/23	96	21[b,d]	95	5[c,d]	23
	3	21/23	91	17[a,b,c,d]	81	6[b,d]	29

[1]Groups: (1) 10% glycerol for 10 min, (2) 25% glycerol for 10 min, (3) 25% glycerol for 5 min, (4) 25% glycerol for 2.5 min; [2]Number of embryos recovered per number of embryos vitrified; [3]Developmental rate to expanded and hatched blastocysts; [a,b,c,d] Values with different superscripts within the same column differ ($p<0.05$).

Table 2. *In vivo* development after vitrification of mouse compact morulas and blastocysts in 45% glycerol.

Stage of development	Control (fresh) embryos						Vitrified embryos					
	Transferred[1]		Implantation sites		Fetuses		Transferred[1]		Implantation sites		Fetuses	
	N	(%)	N	(%)	N	(%)	N	(%)	N	(%)	N	(%)
Compact morula	58	(29)	23[a,A]	(40)	19[a,A]	(33)	51	(28)	20[a,A]	(39)	14[a,A]	(27)
Blastocyst	66	(33)	39[a,B]	(59)	34[a,A]	(52)	56	(32)	8[b,B]	(14)	5[b,B]	(9)
Total	124	(31)	62[a]	(50)	53[a]	(43)	107	(30)	28[b]	(26)	19[b]	(18)

[1]Number (N) indicates the total number of embryos transferred to pregnant recipients; [a,b] Values with different superscripts within the same rows differ ($p<0.05$); [A,B] Values with different superscripts within the same column differ ($p<0.05$).

control embryos (Experiments 1 and 2). The results obtained with vitrified morulas confirmed the close relationship between *in vitro* and *in vivo* survival, whereas the evaluation of survival of vitrified blastocysts after 1 and 48 h of IVC was not a good indicator of embryo viability and *in vivo* development after transfer. Yet again, this substantiates that the transfer of embryos to female recipients is still the final and definitive criterion for the evaluation of embryo viability following freezing [28].

One of the main criteria used in this study for the morphological evaluation of embryo survival after thawing was the process of embryo shrinking during the cryoprotectant dilution, followed by their the re-expansion when in holding medium, which may be indicative of plasma membrane integrity [24,25]. Such ef-

fect may be related to the glycerol permeation into the cell [23], which has a direct relationship with factors such as the cryoprotectant concentration, and time and temperature of exposure [8,11]. Injuries due to either inadequate dehydration prior to freezing, which can lead to the formation of ice crystals in and around cells, or to toxic effects of the solution may occur when those factors are insufficient or in excess. Both events may compromise survival due to membrane and cell injuries or cryoprotectant toxicity. Such effects are not prominent for early stages, since the relative efficacy observed in this study for the compact morula stage was high and fell well within values (81 to 89%) reported by others [1,5,15,22] following the vitrification of mouse embryos at different stages of development under dis-

tinct protocols. The low relative efficacy observed for blastocysts reflects the poor survivability of this stage of development after vitrification in 45% glycerol, which may be a consequence of one or both factors mentioned above, i.e., damage due to ice crystal formation and/or glycerol's toxicity.

More advanced stages of development, such as blastocysts, contain tighter intercellular junctions. At this stage of development, cryoprotectant agents must transverse the cells to reach the blastocoele, preventing damage to the inner surface of cells into that cavity [30]. Consequently, physical damage during freezing may occur due to a slow intercellular flux of water, which increases chances for ice crystal formation within and between cells, reducing viability. Water loss occurs more easily through blastomeres and from the outer surface of the embryo. In this process, water and cryoprotectant must flow in opposite directions, especially for blastocysts.

When water and cryoprotectant fluxes are reduced, more ice crystals may form within cells and into the blastocoele, leading to lesions in the cell membranes of both the inner cell mass (ICM) and the trophectoderm [17]. If such damage is extensive enough to the ICM, a dramatic reduction in development is expected, despite the re-expansion of the blastocoele following freezing. Such feature may explain the discrepancy seen in this study between *in vitro* and *in vivo* survivals after vitrification of blastocysts. In blastocysts, an increase in early mortality may be caused by injuries suffered by the inner cell mass during the process of vitrification or cryoprotectant dilution. Morphologically, embryos may appear viable following 1 h of IVC after thawing, based on the re-expansion of the balstocoelic cavity. However, as damage to the ICM may not be easily detected by morphological evaluation, the transfer of putative viable blastocysts may result in poor *in vivo* development after transfer, as observed in this study.

Embryos from different species or stages of development have distinct levels of tolerance to cryoprotectant agents and to distinct cooling and warming rates during freezing [2,6,8,31]. Such distinctions can also be present between blastomeres in the same embryo [16], which may be directly related to higher losses after cryopreservation of more advanced stages of development [18], as seen for blastocysts in this study. In general, the exposure to 25% glycerol for 5 and 2.5 min (groups 3 and 4, respectively) promoted lower rates of development after 48 hours of IVC, especially for blastocysts and expanded blastocysts. Consequently, the decrease in exposure time to 25% glycerol appeared to have limited pre-dehydration, since the exposure for longer times appeared to have conferred a more appropriate survival post-thawing.

At the blastocyst stage, the amount of water present in the blastocoele, along with its osmotic response, plays the most critical role during freezing, determining the proportion of survival or injuries post-thawing [9,14]. This may also explain the lower survival for blastocysts and expanded blastocysts in this study. Leibo [10] suggested that differences in freezability between stages of embryo development could be explained by their cellular and intercellular structures. Compact morulas and early blastocysts behave osmotically as a single cell when exposed to a hypertonic solution [9], whereas blastocysts and expanded blastocysts do not. The toxic effects of the cryoprotectant solution on survival and development cannot be neglected. In addition, cell permeability to glycerol increases according to embryo development [9]. The presence of two cell lineages with distinct conformations and masses (ICM and trophectoderm), the presence of a fluid-filled cavity (blastocoele), and the higher cell permeability to glycerol in comparison to earlier stages [7] create a paradoxical situation in which a prolonged exposure to glycerol may allow an appropriate cell and cavity dehydration, yet causing toxic effects to the cells, and vice versa. In this context, expanded blastocysts should permeate glycerol more promptly than earlier stages of development, as mentioned above.

Fahy [3] suggested that freezing damage due to high cryoprotectant concentrations are predominantly caused by osmotic mechanisms, as excessive glycerol permeation could cause cell swelling and membrane damage during cryoprotectant dilution following thawing. In fact, the exposure of embryos to the highly concentrated vitrification solutions has been also shown to decrease the *in vivo* survival and the number of viable fetuses following transfer [20]. The toxic effects of the vitrification solutions or the vitrification procedure *per se* may impair the kinetics of development, causing viable embryos to delay development, which may lead to increased implantation failures.

CONCLUSIONS

The *in vitro* survival of mouse embryos after the vitrification demonstrated that the exposure to 10 or 25% glycerol for 2.5, 5, or 10 min at 20°C provided adequate cell dehydration and cryoprotectant effect to compact morulas and blastocysts, but not to expanded

blastocysts, prior to vitrification in 45% glycerol. However, the *in vivo* survival after the vitrification of mouse embryos in 45% glycerol was only effective for the compact morula stage, which attained rates of development similar to controls (fresh embryos). Although the *in vitro* survival following thawing was relatively high and not different from compact morulas, the vitrification of blastocysts at 45% glycerol was detrimental to the subsequent *in vivo* development. A positive association between the *in vitro* survival at 1 and 48 h post-thawing and the *in vivo* development following the vitrification of compact morulas in 45% glycerol, which was not a valid parameter for the vitrified blastocyst stage in the mouse.

SOURCES AND MANUFACTERS

[1]Intervet, The Netherlands.
[2]Organon, Brazil.

REFERENCES

1 **Cseh S., Horlacher W., Brem G., Corselli J., Seregi J., Solti L. & Bailey L. 1999.** Vitrification of mouse embryos in two cryoprotectant solutions. *Theriogenology.* 52: 103-113.

2 **Emiliani S.**, **Van den Bergh M., Vannin A. S., Biramane J. & Englert Y. 2000.** *Human Reproduction.* 15: 905-910.

3 **Fahy G.M. 1986.** The relevance of cryoprotectant "toxicity" to cryobiology. *Cryobiology.* 23: 1-13.

4 **Fahy G.M., McFarlane D.R., Angell C.A. & Meryman H.T. 1984.** Vitrification as an approach to cryopreservation. *Cryobiology.* 21: 407-426.

5 **Friedler S., Shen E. & Lamb E.J. 1987.** Cryopreservation of mouse 2-Cell embryos and ova by vitrification: Methodologic Studies. *Fertility and Sterility.* 48: 306-314.

6 **Ishida G.M., Saito H., Ohta N., Takahashi T., Ito M.M., Saito T., Nakahara K. & Hiroi M. 1997.** *Human Reproduction.* 12: 1259-1262.

7 **Jackowski S.C. & Leibo S.P. 1976.** Response of fertilized mouse ova to freezing and thawing as a function of permeation by glycerol. *Cryobiology.* 13: 646.

8 **Kasai M. 2002.** Advances in the cryopreservation of mammalian oocytes and embryos: Development of ultrarapid vitrification. *Reproductive Medicine and Biology.*1:1-9.

9 **Lehn-Jensen H. & Rall W.F. 1983.** Cryomicroscopic observations of catle embryos during freezing and thawing. *Theriogenology.* 19: 263-277.

10 **Leibo S.P. 1977.** Fundamental cryobiology of mouse ova and embryos. *CIBA Foundation Symposium.* 52: 69-96.

11 **Leibo S.P. & Mazur P. 1978.** Methods for the preservation of mammalian embryos by freezing. In: Daniel Jr. J.C. (Ed). *Methods in Mammalian Embryology II.* London: Academic Press, pp.197-201.

12 **Liebermann J., Nawroth F., Isachenko V., Isachenko E., Rahimi G. & Tucker M.J. 2002.** Potential importance of vitrification in reproductive medicine. *Biology of Reproduction.* 67: 1671-1680.

13 **Luyet B.J. 1937.** The vitrification of organic colloids and of protoplasm. *Biodynamica.* 1: 1-14.

14 **Massip A., Van der Zwalmen P. & Leroy F. 1984.** Effects of stage of development on survival of mouse embryos frozen-thawed rapidly. *Criobiology.* 21: 574-577.

15 **Massip A., Van der Zwalmen P., Scheffen B. & Ectors F. 1986.** La vitrification, methode d'avenir pour la cryoprotection des embryons. In: Menezo Y. & Merieux C. (Eds). *Seminaire sur la Congelation des Embryons et des Ovocytes.* Annecy: Fondation Marcel Merieux, pp.179-194.

16 **Maurer R.R. 1978.** Freezing mammalian embryos: a review of the techniques. *Theriogenology.* 9: 45-69.

17 **Mazur P. 1970.** Cryobiology: the freezing of biological systems. *Science.* 168: 939-949.

18 **Mohr L.H. & Trounson A.O. 1981.** Structural changes associated with freezing of bovine embryos. *Biology of Reproduction.* 25: 1009-1025.

19 **Mukaida T., Wada S., Takahashi K., Pedro P.B., An T.Z. & Kasai M. 1998.** Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Human Reproduction.* 13: 2874-2879.

20 **Rall W.F., Wood M.J., Kirby C. & Whittingham D.G. 1987.** Development of mouse embryos cryopreserved by vitrification. *Journal of Reproduction and Fertility.* 80: 499-504.

21 **Roberston I. & Nelson R.E. 1998.** Certification and identification of the embryo. In: Stringfellow D.A. & Seidel S.M. (Eds). *Manual of the International Embryo Transfer Society.* 3rd edn. Savoy: IETS, Inc., pp.103-134.

22 **Scheffen B., Van der Zwalmen P. & Massip A. 1986.** A simple and efficient procedure for preservation of mouse embryos by vitrification. *Cryo-Letters.* 7: 260-269.