The effect of cryopreservation on the lethal mutation rate in *Drosophila melanogaster*

DAVID HOULE[1]*, ALEXEY S. KONDRASHOV[2], LEV YU. YAMPOLSKY[2], SHANNON CALDWELL[3] AND PETER L. STEPONKUS[3]

[1] *Department of Zoology, University of Toronto, 110 St George Street, Toronto, Ontario, M5S 3G5, Canada*
[2] *Section of Ecology and Systematics, Cornell University, Ithaca, NY 14853, USA*
[3] *Department of Soil, Crop, and Atmospheric Sciences, Cornell University, Ithaca, NY 14853, USA*

(*Received 30 January 1997 and in revised form 24 March 1997*)

Summary

Although cryopreservation is routinely used for the storage of a range of biological organisms, few studies have been conducted to determine whether cryopreservation increases the frequency of mutation. A procedure for the cryopreservation of *Drosophila melanogaster* embryos has recently been developed. Cryopreservation of *D. melanogaster* is of special interest to geneticists and evolutionary biologists because it would make it possible to assay control and experimental populations simultaneously during long-term studies. Before cryopreserved embryos can be used for such studies, it is first necessary to show that cryopreservation is not mutagenic. We tested for mutagenic effects of cryopreservation in *D. melanogaster* embryos with an X-linked, recessive lethal assay. The mutation rates of cryopreserved and control flies were not significantly different. We can be 95% certain that cryopreservation does not increase mutation by a factor greater than 2·39. This is the first quantitative estimate of the mutagenic effect of cryopreservation on the germ line of a metazoan. The results are reassuring when considering the genetic impact of cryopreservation on mammalian gametes and embryos.

1. Introduction

The ability to store viable specimens of biological organisms for long periods is tremendously valuable for geneticists, medical scientists, plant and animal breeders, and evolutionary biologists. Historically, this was only possible with organisms that had evolved a long-lived resting stage, such as seeds or spores. The development of cryopreservation procedures has now extended the range of organisms that can be preserved over long periods to include bacteria, fungi, plants, nematodes, and embryos of many mammals, including mice, sheep and cattle. Cryopreservation is now routinely used to preserve unique, elite or endangered genetic lines in many of these organisms. In addition, cryopreserved human embryos and gametes are frequently used in treatments for infertility (Tucker *et al.*, 1995).

Recently, a procedure for the cryopreservation of embryos of the fruit fly, *Drosophila melanogaster*, has been developed (Steponkus *et al.*, 1990, 1993; Mazur *et al.*, 1992; Steponkus & Caldwell, 1993). The ability to cryopreserve *D. melanogaster* is important to geneticists, who maintain tens of thousands of genetically distinct stocks by serial transfer of flies every month. Not only are the lines vulnerable to accidental loss or contamination, but selection, drift and mutation make it virtually certain that stocks will not retain the same genotype over time (Bailey, 1977). Cryopreservation can also greatly aid multi-generation studies of mutation and evolution by allowing simultaneous comparison of ancestral and derived populations.

Before cryopreserved embryos can be used for these purposes, it is important to establish both that the cryopreservation process is not mutagenic and that mutations do not accumulate during storage of cryopreserved material. Few studies of the effects of cryopreservation on mutation rates have been performed in metazoans. There has been one study of the effect of cryopreservation on germ line mutation rates in mice; however, its authors did not report quantitative results (Lyon *et al.*, 1981). The only other directly relevant study we are aware of showed that cryopreservation of human T-cells did not increase the mutation rate at a selectable locus (McGinnis *et*

* Corresponding author. Tel: +1(416)-978–1014. Fax: +1(416) 978–8532. e-mail: dhoule@zoo.toronto.edu.

al., 1990). This dearth of information is largely due to the difficulties involved in large genetic screens in vertebrates. A much larger number of studies have looked for indirect evidence of genetic damage, such as chromosomal anomalies. Enough of these studies have found evidence of such damage to be a cause for concern (Rudd *et al*., 1989; Schmidt-Preuss *et al*., 1990; Casati *et al*., 1992; Bouquet *et al*., 1993). The advent of cryopreservation in *D. melanogaster* offers a good opportunity to perform large-scale genetic analyses of the effects of such techniques in a complex metazoan.

In this paper we report the results of experiments designed to test for mutagenic effects of cryopreservation on *D. melanogaster* embryos, using the mutation rate to X-linked recessive lethals as an assay. Our primary goal was to determine whether the cryopreservation procedure developed for *D. melanogaster* (Steponkus & Caldwell, 1993; Steponkus *et al*., 1993) is mutagenic. We addressed this question by subjecting embryos to the cryopreservation procedure, then recovering them after only 10–60 min in liquid nitrogen. In addition, we recovered other embryos after storage of up to 18 months in liquid nitrogen to provide information on mutation during storage. To our knowledge, this is the longest period that individual *D. melanogaster* have been cryopreserved.

2. Materials and methods

Mutation rates were determined in inbred lines derived from a replicate of the Ives (IV) population (Charlesworth & Charlesworth, 1985), marked with the visible mutant *ebony* (*e*). This allele occurred spontaneously in the IV population in 1992, and the population was made homozygous for *e* by mass back-crossing to Ives and selection for *e* in four cycles. Inbred IV*e* lines were obtained by 30 generations of half- and full-sib mating; two of these lines, IV-28 and IV-39, were used in the present experiment.

Flies were reared on a sucrose, cornflour and brewer's yeast medium, with propionic acid added to reduce the growth of microorganisms. An alternative medium containing the anti-fungal agent Nipagin (methyl 4-hydroxybenzoate) at 0·03% proved lethal to cryopreserved larvae. All rearing took place at 25 °C with a 12 h photoperiod. Eggs were harvested from cages containing approximately 5000 adult flies, most of which were less than 10 days post-eclosion. Embryos to be used for the control and the 'cryopreservation' treatments were obtained from the same cages over the same 4 day period; embryos for the 'cryopreservation + storage' treatment were obtained in the subsequent week. Embryos were collected on plates coated with live baker's yeast placed in the cages for 1 h. Control eggs were allowed to develop on the collection plates at 25 °C, and the emerging larvae were transferred from collection plates to yeasted vials.

Prior to cryopreservation, embryos were allowed to develop until they were 13·5–14·5 h old, then dechorionated with a sodium hypochlorite solution. The waxy layer of the vitelline membrane was then removed by sequential treatment with isopropanol and hexane. The permeabilized embryos were equilibrated in a 2·1 M solution of ethylene glycol for 20 min at 25 °C, and dehydrated in a solution containing 42 wt% ethylene glycol + 6 wt% BD20 solutes for 10 min at 0 °C. The embryos were then plunged into nitrogen slush at −205 °C (cooling rate 400 °C/s) to achieve vitrification. Embryos were stored in liquid nitrogen at −196 °C.

Recovery from liquid nitrogen storage was accomplished by rapid warming of the embryos in a 1 M sucrose solution at room temperature, followed by a three-step dilution of the vitrification solution. Embryos were then maintained at high humidity under mineral oil until hatching. Larvae were individually recovered from the mineral oil and placed on fresh medium to which dried live baker's yeast was added. This procedure has been optimized for survival of an Oregon-R stock, with which it reliably yields survival to adulthood of 45% of the cryopreserved embryos. Additional details on this procedure may be found elsewhere (Steponkus & Caldwell, 1993; Steponkus *et al*., 1993).

In the 'cryopreservation' treatment, embryos remained in liquid nitrogen for 10–60 min. In the 'cryopreservation + storage' treatment, some embryos were recovered after 14 months of storage. The survival of these embryos was low (see Section 3), so an additional set of recoveries was made 4 months later. The 14 and 18 month recoveries yielded indistinguishable lethal assay results; they are lumped as the 'cryopreservation + storage' treatment in Table 1.

In each treatment, virgin parental (P) females were collected as they eclosed, and mated to males carrying the X-chromosome balancer FM6. Virgin F_1 daughters were then back-crossed to FM6 males. At the time of cryopreservation, the female germ line consists of approximately 16 cells (Wieschaus & Szabad, 1979). For the control and 'cryopreservation' treatments, we assayed up to 16 daughters of each P female to allow for the possibility of detecting mutational clusters from the 16-cell stage, without undue resampling from the same stem cells. For the 'cryopreservation + storage' treatment, we assayed up to 20 daughters of each P female. A lack of wild-type males among the F_2 offspring indicates the presence of a recessive lethal.

All putative lethals were retained, and the offspring of approximately 10 F_2 females were assayed to verify the presence of a lethal. If additional F_1 flies were available, 10–20 additional sisters of a lethal-bearing F_1 female were crossed to FM6 to obtain additional information on the frequency of the lethal in the germ line of the P female. Crosses for the lethal assay were

https://doi.org/10.1017/S0016672397002760 Published online by Cambridge University Press

carried out in two laboratories. No differences in mutation rates were detected between laboratories, so these data were pooled.

Description of mutant strains may be found in Lindsley & Zimm (1992).

3. RESULTS

A total of approximately 6600 embryos were cryopreserved and recovered in this study. The survival of the two genotypes differed following cryopreservation. In the 'cryopreservation' treatment, in which embryos were recovered after no more than an hour of storage in liquid nitrogen, 65·4% of the embryos of line IV-39 hatched and 29% of the recovered larvae eclosed; with IV-28, 30·6% of the embryos hatched and 9% of the recovered larvae eclosed. Hatching percentages for untreated embryos of IV-39 and IV-28 were 91 and 74%, respectively. With both lines, 85–90% of untreated larvae normally survive to eclosion. All the IV-28 flies were used in the immediate treatment, as the survival of this line proved so low. Unexpectedly, the survival of embryos in the 'cryopreservation + storage' treatments was lower. In the 14 month recovery, 55·5% of the IV-39 eggs hatched, but only 9·7% of the recovered larvae survived to eclosion. At 18 months, 50·3% of the eggs hatched and 8·9% of the recovered larvae survived to eclosion.

Examination of the larval vials from all recovery times revealed that a substantial proportion of the mortality took place in first instar larvae. Many of these doomed larvae had crawled onto the sides of the vial rather than remaining on the food. Mortality was also observed during other instars and during the pupal phase.

We chose to assay the mutation rate for loci on the X-chromosome to take advantage of the fact that males are hemizygous for this chromosome. We assayed mutation rate only in females subjected to cryopreservation because we were concerned that selection among cell lines in a hemizygous male could lead to greater loss of lethal mutants in the cryopreservation treatments, given the encompassing nature of the cryopreservation procedure. The use of females has a cost in that we cannot be sure that the treated embryos did not carry a pre-existing lethal, and hence the occurrence of mutational clusters is more difficult to interpret. The use of the X-chromosome has a cost in that there is no straightforward way to determine the allelism of the recovered lethals.

Results of the mutation assay are shown in Table 1. Most of the data was obtained from IV-39 because of the low survival of IV-28. Clusters of multiple lethals were recovered in the offspring of five IV-39 P females, as shown in Table 1. All other lethals were recovered from different P females. The five clusters each contained large numbers of lethals: 13/36, 12/30, 16/30, 7/19 and 9/23 of the chromosomes assayed. In each case, these ratios are not significantly different from the 1/2 expected if these were the result of heterozygosity of the P female for a pre-existing lethal. We think that it is unlikely that these clusters were the result of cryopreservation. Cryopreservation took place when there were approximately 16 cells in the developing germ line (Wieschaus & Szabad, 1979), and hence the expectation is that clusters due to cryopreservation should have made up a small proportion of the total recovered in each case, rather than close to half. The complete absence of any small clusters argues strongly against cryopreservation as the cause.

We begin our analysis by assuming that the clusters of lethals are due to heterozygosity of the zygotes, and hence unrelated to cryopreservation treatment. We assumed that each F_1 female assayed represented an independent recovery of a treated or control X-chromosome. Mutation rates were in the range typical for the X-chromosome. Previous studies have shown that laboratory stocks have an average X-linked lethal mutation rate of $1{\cdot}6 \times 10^{-3}$, and wild caught flies a rate of $2{\cdot}5 \times 10^{-3}$, although individual experiments show a

Table 1. *Mutation rates in cryopreserved and control* D. melanogaster.

	Excluding lethal clusters				
Line	P females	Chromosomes tested	Lethals	Rate $\times 10^3$	Lethal clusters
IV-39					
Control	308	4334	5	1·15	1
Cryopreservation	215	3063	5	1·63	3
Cryopreservation + storage	92	1513	1	0·66	1
Total	615	8910	11	1·24	5
IV-28					
Control	104	1609	5	3·11	0
Cryopreservation	29	437	1	2·29	0
Total	133	2046	6	2·93	0

https://doi.org/10.1017/S0016672397002760 Published online by Cambridge University Press

wide range around these values (Woodruff *et al.*, 1983). The risk of lethal mutation was analysed by maximum-likelihood logistic analysis in the SAS program Catmod (SAS Institute, 1990*a*). Analysis of the data, excluding the lethal clusters, with cryopreservation treatment (control, 'cryopreservation' and 'cryopreservation + storage') and line as main effects showed that the effect of cryopreservation was not significant ($\chi^2 = 0{\cdot}57$, 2 d.f., $P = 0{\cdot}75$), although the effect of line was closer to significance ($\chi^2 = 2{\cdot}36$, 1 d.f., $P = 0{\cdot}12$). Thus, our data suggest that the two genotypes may differ in their mutation rates, but provide no evidence that cryopreservation or storage of embryos is mutagenic. Separate analysis of just the 'cryopreservation' and 'cryopreservation + storage' treatments confirmed that they were also not significantly different from each other ($\chi^2 = 0{\cdot}69$, 1 d.f., $P = 0{\cdot}41$).

To calculate a meaningful summary relative risk statistic for the effect of cryopreservation, we lumped the 'cryopreservation' and 'cryopreservation + storage' treatments for line IV-39. Logistic analysis of the pooled data again showed that neither cryopreservation ($\chi^2 = 0{\cdot}00$, 1 d.f., $P = 0{\cdot}99$) nor line ($\chi^2 = 2{\cdot}71$, 1 d.f., $P = 0{\cdot}10$) affected mutation rate. Using the Cochran–Mantel–Haenszel procedure (Landis *et al.*, 1978; SAS Institute, 1990*b*), we estimate that the relative risk of a lethal mutation is increased by cryopreservation by a factor of 1·003, with an upper, one-sided 95% confidence limit of 2·39.

If we assume that the mutational clusters arose during the experimental generation, chromosomes are no longer independent with respect to cryopreservation treatment, and we can only test whether the probability that any lethals were found in the offspring of each parental female differs with treatment. Lumping the 'cryopreservation' and 'cryopreservation + storage' treatments, there is again no evidence for an effect of treatment ($\chi^2 = 0{\cdot}57$, 1 d.f., $P = 0{\cdot}45$) or line ($\chi^2 = 1{\cdot}79$, 1 d.f., $P = 0{\cdot}18$). For line IV-39 alone there is also no evidence of a treatment effect ($\chi^2 = 1{\cdot}00$, 1 d.f., $P = 0{\cdot}32$).

4. DISCUSSION

We have shown that neither the cryopreservation procedure nor long-term storage affected the rate of recessive lethal mutation, within the limits of the power of our assay. Our finding is an essential step in justifying the use of cryopreservation of *D. melanogaster* for genetic and evolutionary studies. The X-chromosome of *D. melanogaster* contains about 20% of the genome and approximately 500–1000 loci that can mutate to a recessive lethal phenotype. This assay should therefore detect a wide range of genetic damage (Vogel *et al.*, 1981). Given this large sample of the genome, it is likely that other types of mutations, such as dominant lethals and non-lethal lesions, also occur at near the background rate.

Our results relieve a number of concerns about the potential for mutagenic effects as a direct result of our cryopreservation procedure. Several steps are potentially mutagenic, such as exposure to high cytosolic concentrations of ethylene glycol, and the extreme dehydration that is a requisite for vitrification. Enhanced mutation rates of dried versus wet cells, spores and seeds have been reported in a variety of organisms (e.g. Ashwood-Smith & Grant, 1976). Two previous studies have suggested that cold treatment of embryos (Birkina, 1938) or adults (Kerkis, 1941) increases the frequency of sex-linked recessive lethals, although a much larger study subsequently found no evidence of such effects (Rendel & Sheldon, 1956).

In addition, our study demonstrates that long-term storage of *D. melanogaster* embryos is feasible. In previous studies (P. L. Steponkus and S. Caldwell, unpublished results), the longest time that *D. melanogaster* embryos were stored in liquid nitrogen was 1 month. In the present study, the embryos were stored in liquid nitrogen for 18 months, which is long enough to be useful for evolutionary and mutational studies.

Although there was a substantial decrease in the percentage of larvae that eclosed in the 'cryopreservation + storage' treatment as compared with the 'cryopreservation' treatment, we do not believe that this was a consequence of long-term storage *per se*. With mammalian gametes and embryos there is very little, if any, decrease in survival during storage in liquid nitrogen (Glenister *et al.*, 1990; Leibo *et al.*, 1994). In a previous study of *D. melanogaster* Oregon-R P2 embryos, there was no decline in survival (either percentage hatching or percentage eclosion) after storage in liquid nitrogen for 1 month (P. L. Steponkus and S. Caldwell, unpublished results). We suspect that the large decrease in the percentage eclosion of the embryos in the 'cryopreservation + storage' treatment was a consequence of inadequate control over the age (developmental stage) of the embryos that were used for cryopreservation. Embryos that were used for the 'cryopreservation + storage' treatment were cryopreserved several days after those that were used for the 'cryopreservation' treatment and were possibly at a slightly different developmental stage. Previous studies have shown embryo survival is strongly dependent on the age (developmental stage) of the embryos that are used for cryopreservation (Mazur *et al.*, 1992; Steponkus & Caldwell, 1992). With Oregon-R P2, there is a very narrow range in the optimum age of embryos that yields maximum survival, and the use of embryos that are as little as 15 min older than the maximum results in a sharp decline in survival (Steponkus *et al.*, 1996). For example, maximum survival (55% hatching) was attained for 13·75–14·75 h embryos; however, survival decreased to 20% hatching for 14–15 h embryos. Furthermore, probability of eclosion is even more sensitive to developmental timing. The results of the present study are consistent with these observations, i.e. although

https://doi.org/10.1017/S0016672397002760 Published online by Cambridge University Press

there was a substantial decrease in the percentage eclosion, there was only a relatively small decrease in the percentage hatching. Thus, it is possible that the decreased eclosion percentage in the 'cryopreservation + storage' treatment was a result of the embryos being slightly older at the time of cryopreservation than those that were used in the 'cryopreservation' treatment. Clearly, further experiments are necessary to resolve this matter.

Our study is the first to give a quantitative estimate of the mutation rate during cryopreservation and storage of an intact metazoan and therefore provides some reassurance concerning the effects of cryopreservation in vertebrate systems in which broad-based assays of mutagenesis are far more difficult. This must be qualified of course, as arthropods are very distantly related to the vertebrates and there are important differences between the cryopreservation procedure developed for *D. melanogaster* embryos and those used for other organisms (Steponkus *et al*., 1990, 1993; Steponkus & Caldwell, 1993).

We thank B. Morikawa, T. Tocheva, J.-A. An, K. Westlake, S. Carvalho and C. Wright for assistance. This work was funded in part by NSERC Canada.

References

Ashwood-Smith, M. J. & Grant, E. (1976). Mutation induction in bacteria by freeze-drying. *Cryobiology* **13**, 206–213.

Bailey, D. W. (1977). Genetic drift: the problem and its possible solution by frozen-embryo storage. In *The Freezing of Mammalian Embryos* (Ciba Foundation Symposium 52), pp. 291–299. Elsevier: Amsterdam.

Birkina, B. N. (1938). The effect of low temperature on the mutation process in *Drosophila melanogaster*. *Biologicheskii Zhurnal* **7**, 653–660.

Bouquet, M., Selva, J. & Auroux, M. (1993). Cryopreservation of mouse oocytes: mutagenic effects in the embryo? *Biology of Reproduction* **49**, 764–769.

Casati, A., Stefanini, M., Giorgi, R. & Nuzzo, F. (1992). Different rate of chromosome breakage in human fibroblast strains after storage in liquid nitrogen. *Mutation Research* **275**, 7–11.

Charlesworth, B. & Charlesworth, D. (1985). Genetic variation in recombination in *Drosophila*. I. Responses to selection and preliminary genetic analysis. *Heredity* **54**, 71–83.

Glenister, P. H., Whittingham, D. G. & Wood, M. J. (1990). Genome cryopreservation: a valuable contribution to mammalian genetic research. *Genetical Research* **56**, 253–258.

Kerkis, J. (1941). The effect of low temperature on the mutation frequency in *D. melanogaster* with consideration about the causes of mutations in nature. *Drosophila Information Service* **15**, 25.

Landis, R. J., Heyman, E. R. & Koch, G. G. (1978). Average partial association in three-way contingency tables: a review and discussion of alternative tests. *International Statistical Review* **46**, 237–254.

Leibo, S. P., Semple, M. E. & Kroetsch, T. G. (1994). *In vitro* fertilization of oocytes by 37-year-old cryopreserved bovine spermatozoa. *Theriogenology* **42**, 1257–1262.

Lindsley, D. L. & Zimm, G. (1992). *The Genome of* Drosophila melanogaster. San Diego: Academic Press.

Lyon, M. F., Glenister, P. H. & Whittingham, D. G. (1981). Long term viability of embryos stored under irradiation. In *Frozen Storage of Laboratory Animals* (ed. G. H. Zeilmaker), pp. 139–147. Stuttgart: G. Fischer.

Mazur, P., Cole, K. W., Hall, J. W., Schreuders, P. D. & Mahowald, A. P. (1992). Cryobiological preservation of *Drosophila* embryos. *Science* **258**, 1932–1935.

McGinnis, M. J., Falta, M. T., Sullivan, L. M. & Albertini, R. J. (1990). *In vivo hprt* mutant frequencies in T-cells of normal human newborns. *Mutation Research* **240**, 117–126.

Rendel, J. M. & Sheldon, B. L. (1956). The effect of cold treatment on mutation in *Drosophila melanogaster*. *Australian Journal of Agricultural Research* **7**, 566–573.

Rudd, N. L., Hoar, D. I., Williams, S. E. & Hennig, V. G. G. (1989). Genotype and cryopreservation process affect the levels of aneuploidy and chromosome breakage in cultured human fibroblasts. *Genome* **32**, 196–202.

SAS Institute (1990*a*). *SAS Procedures Guide*, *Version 6*, 3rd edn. Cary, NC: SAS Institute.

SAS Institute (1990*b*). *SAS/STAT User's Guide*, *Version 6*, 4th edn. Cary, NC: SAS Institute.

Schmidt-Preuss, U., Roser, M., Weichenthal, M. & Ruediger, H. W. (1990). Elevated frequencies of micronuclei in cultured human fibroblasts after freezing and thawing. *Mutation Research* **241**, 279–282.

Steponkus, P. L. & Caldwell, S. C. (1992). Cryopreservation of *Drosophila melanogaster* embryos by vitrification. *Cryobiology* **29**, 763–764.

Steponkus, P. L. & Caldwell, S. (1993). An optimized procedure for the cryopreservation of *Drosophila melanogaster* embryos. *Cryo-Letters* **14**, 375–380.

Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T. & MacIntyre, R. J. (1990). Cryopreservation of *Drosophila melanogaster* embryos. *Nature* **345**, 170–172.

Steponkus, P. L., Caldwell, S. & Wisniewski, C. (1993). Cryopreservation of *Drosophila melanogaster* embryos: optimization of the procedure for maximum survival. *Cryobiology* **30**, 617.

Steponkus, P. L., Caldwell, S. C., Myers, S. P. & Cicero, M. (1996). Cryopreservation of *Drosophila melanogaster* embryos. In *Advances in Low-Temperature Biology*, vol. 3 (ed. P. L. Steponkus), pp. 257–316. London: JAI Press.

Tucker, M. J., Morton, P. C., Sweitzer, C. L. & Wright, G. (1995). Cryopreservation of human embryos and oocytes. *Current Opinion in Obstetrics and Gynecology* **38**, 1–5.

Vogel, E., Schalet, A., Lee, W. R. & Würgler, F. (1981). Mutagenicity of selected chemicals in *Drosophila*. In *Comparative Chemical Mutagenesis* (ed. F. J. De Serres & M. D. Shelby), pp. 175–256. New York: Plenum Press.

Wieschaus, E. & Szabad, J. (1979). The development and function of the female germ line in *Drosophila melanogaster*: a cell lineage study. *Developmental Biology* **68**, 29–46.

Woodruff, R. C., Slatko, B. E. & Thompson, J. N., Jr (1983). Factors affecting mutation rates in natural populations. In *The Genetics and Biology of Drosophila*, vol. 3c (ed. M. Ashburner, H. L. Carson & J. N. Thompson Jr), pp. 37–124. London: Academic Press.