a whole because of a continual replacement regimen[3,4]. This assumption is also based on some known observations in which several sea anemones were maintained in aquaria for a very long time, and underwent no obvious change during 80–90 years of continuous observations[3]. In contrast, few indications point to the existence of aging and that individuals of coelenterates really die. In one case, a specimen of the sea anemone *Sagartia troglodytes* was kept in captivity for 66 years. This animal died naturally, after appearing to become weaker for several months[19].

In the scleractinian corals there is no direct evidence for the existence of old age, decay and natural death. Only in one case was it suggested that reproduction in corals could be followed by subsequent senescence and death[20]. Thus, it is generally believed that within a colony 'death and life are going on together by asexual reproduction and cease of the old polyps'[21]. The present study, therefore, provides evidence for the first time that the death of a coral colony could follow natural decremental processes and not only result from storm activities, sedimentation, changes in salinity or temperature, low tides, changes in light intensities, predation, competition[22] and disease[23]. This brings us face to face with one of the most difficult problems in physiology; the nature of the origin of death in corals, which leads to the changes recorded in the calcification and the reproductive processes. We conclude that as in higher multicellular animals, corals accumulate physiological failures which lead to a natural death of the whole colony (which is different form the partial mortality recorded in the past[18]). These failures could also be an indication of the senescence or aging processes of corals. The question of the universality of the aging process, and the question of whether all organisms age by essentially the same biological process, are critical in choosing an organism for research into aging in an attempt to obtain an understanding of human aging processes[24]. Although the present study characterizes processes occurring only at the level of the organism it could be of help in understanding aging phenomena. Further work is needed to elucidate the dying processes at other biological organization levels in corals such as in the tissue layer, and at the cellular, subcellular and molecular levels.

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A mitochondrial DNA polymorphism in honeybees (*Apis mellifera* L.)

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Summary. Mitochondrial DNA (mtDNA), isolated from worker honeybee larvae, was digested by each of seven 6-base restriction enzymes. Only one enzyme (*Bgl* II) showed a mtDNA difference between the three tested races (*Apis mellifera carcia, A.m. ligustica, A.m. caucasica*). Both *A.m. carnica* and *A.m. ligustica* showed the same pattern, differing from *A.m. caucasica*. The degree of fragment pattern similarity revealed that there is only a small level of mtDNA variation between the three races tested. This is in line with previous investigations of enzyme polymorphisms.
Key words. Mitochondrial DNA; genetic variation; *Apis mellifera* L.; male haploidy; cytoplasmic inheritance.

The analyses of polymorphisms in nuclear DNA, and in extranuclear DNA, is an important technique for describing variation in populations. In population genetic studies, genomic variation has usually been documented by isozyme studies whereas extranuclear DNA variability is mainly revealed by restriction enzyme analysis of mitochondrial DNA (mtDNA). Both methods are useful for discriminating between different populations and in tracing the evolution of natural diploid populations.

In most hymenopteran populations, which are male-haploid, there are problems because of limited isozyme polymorphisms. Theoretical approaches indicate that the potential for genomic variation in haplo-diploid populations should be reduced in comparison with diploid populations[1-3]. The findings for honeybees are consistent with this view, in that honeybee isozyme polymorphisms seem to be extremely rare[4-7], with very few polymorphic enzyme systems documented for *Apis mellifera* L.[8-14]. However the potential of mtDNA variation should not be reduced in male-haploid populations. Because mitochondria are thought to be inherited maternally[15], there should be no effect of haploid males, and the principles of mtDNA transmission

should be identical in both male-haploid and diploid populations. Because mitochondrial variation is a useful discriminator in diploid populations, a study on mtDNA might reveal new sources of variation within honeybee populations. It therefore may also provide a new approach to estimating relationships between different species and subspecies of *Apis*, and other Hymenoptera in general.

Materials and methods. Worker brood and adult bees were studied of three different races of the honeybee, *Apis mellifera ligustica, A. mellifera carnica* and *A. mellifera caucasica*. The lines were stocks, maintained by artificial insemination at the Hawkesbury Agricultural College, Richmond (New South Wales). Morphometric analysis[16] of 34 characters did not reveal any hybridization between the lines and all three races had the race-typical characteristics. Capped worker brood was taken from nine different colonies, the cells were uncapped and prepupae as well as unpigmented pupae (20 g per sample, 4 samples per colony) were collected. Brood was also allowed to emerge in an incubator (36 °C, 60% relative humidity). Thoraces of freshly emerged workers (20 g per sample) were cut for 1 min in a Waring blender after adding an equal volume of prechilled sucrose-TE-buffer (0.03 M Tris, 0.25 M sucrose, 0.01 M EDTA, pH 7.6).

Each sample was homogenized in a Potter-Elvehjem homogenizer with a teflon pestle after adding an additional 20 ml of sucrose-TE-buffer. The homogenate was centrifuged at 700 × g_{av}, at 4 °C for 20 min in a fixed angle rotor (Sorvall, SS34) to pellet debris and nuclei. The supernatant was centrifuged again under the same conditions, the first pellet was either discarded or used for nuclear DNA isolation. The remaining supernatant was centrifuged at 11,000 × g_{av} for 20 min and a first crude mitochondrial pellet was obtained. The pellet was resuspended in sucrose-TE-buffer and spun again under the previous conditions. To purify the mitochondrial fraction, the pellet was layered on top of a step-wise sucrose gradient (1.0 M/1.7 M in TE buffer) and centrifuged for at least 1 h at 100,000 × g_{av} in an ultracentrifuge with a swing-out rotor (Beckman SW41). Enzyme activity analysis (succinate dehydrogenase) revealed that the mitochondrial fraction sedimented to the top of the 1.7 M sucrose. This fraction was resuspended in STE-buffer (0.1 M NaCl, 0.05 M Tris, 0.01 M EDTA, pH 8), sodium dodecyl sulfate was added to 1% (w/v) final concentration and the suspension shaken for 5 min to dissolve membranes. Protein in the sample was phenol-extracted twice by adding a double volume of buffer-saturated phenol (pH 8.0). After each extraction the sample was spun for 15 min at 8000 × g_{av} to separate the aqueous and phenol phases. The total volume of the aqueous phase was reduced to 400 µl with several butan-1-ol extractions. Residual phenol was removed by a double ether extraction (1 ml ether each). Sodium acetate (pH 6.0) was added to a final concentration of 0.3 M and the mtDNA precipitated by addition of 1 ml 95% (v/v) ethanol followed by standing for 20 min at −70 °C. The mtDNA was pelleted at 12,000 × g in an Eppendorf centrifuge and washed with 80% (v/v) ethanol. The sample was dried under vacuum for 10 min and dissolved in 100 µl TE buffer (0.01 M Tris, 0.01 M EDTA, pH 8.0). The samples were treated with DNAase-free RNAase and again phenol-extracted and ethanol-precipitated as described above. Samples (5 µl) were electrophoresed on a horizontal mini-gel (9 cm × 6 cm, 20 ml, 1% agarose in TAE-buffer: 0.04 M Tris, 0.002 M EDTA, with acetic acid to pH 8.0) at 50 V, for 2 h and stained with ethidium bromide to assess the purity of the preparation. The purified mtDNA samples were stored at −20 °C in 100 µl TE buffer.

Restriction digests were carried out according to the manufacturer's recommendations using about 10 ng mtDNA and 1 U enzyme per reaction. The samples were incubated at 37 °C for 3 h. The DNA fractions were then end-labeled using 1 µCi of (α^{32}P)dATP or (α^{32}P)dCTP (according to the restriction site). Unlabeled deoxy-nucleoside triphosphates (dTTP, dGTP, dCTP or dATP respectively) were added (0.5 mM final concentration) and the reaction was started by addition of 0.3 U DNA polymerase 1 (Klenow fragment). The samples were incubated for 20 min at 25 °C, and one-third volume of loading buffer (60% sucrose, 0.08% bromophenol blue, 0.05 M EDTA) was added and the samples were placed into a submarine horizontal 1% agarose gel (20 cm × 20 cm, 100 ml). Electrophoresis was performed for 12 h at 20 V. *Hind* III or *Bcl* I digests of phage λ DNA were used as size standards. The gel was dried and autoradiographed using Fuji medical X-ray film (exposure time 12–24 h at −70 °C).

Results. We did not find it useful to extract mtDNA from flight muscle mitochondria. Because of the high number of mitochondria in that tissue one might expect a good mitochondrial yield.

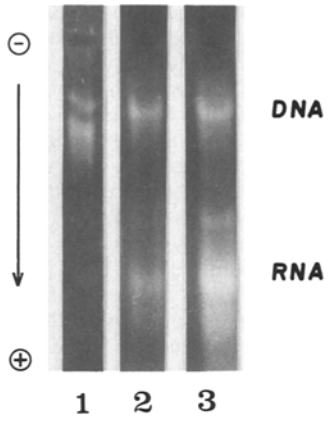

Figure 1. Electropherogram of a 1% agarose gel stained with ethidium bromide and photographed under UV light (260 nm). Larval (2) and pupal (3) preparations of mtDNA show a clear single DNA band, whereas the preparation from adult flight muscle (1) displays the mtDNA fraction on top of a large background smear of genomic DNA. The RNA bands in 2 and 3 disappear after RNAase treatment.

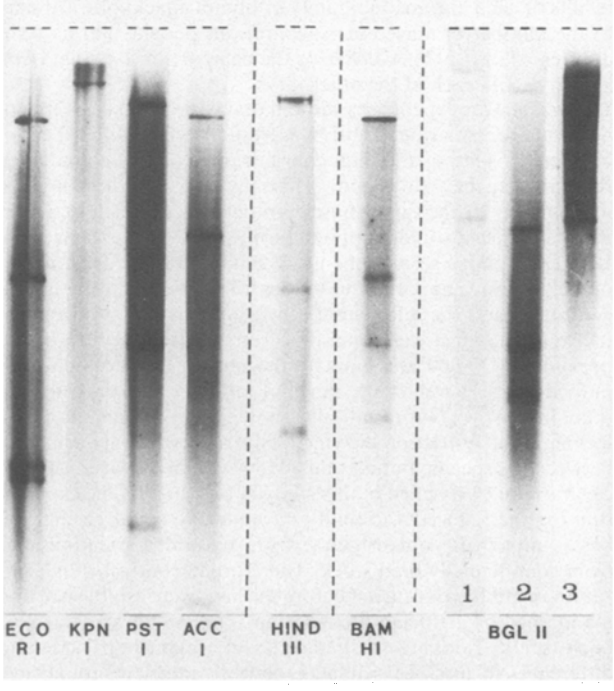

Figure 2. Banding pattern of mtDNA after digestion with seven different restriction enzymes (lanes within dotted lines ran on the same gel). Only *Bgl* II shows a difference between the three tested races. Lane 1: *A. mellifera carnica;* lane 2: *A. m. caucasica;* lane 3: *A. m. ligustica.*

Table 1. Size of mtDNA fragments (1000 base pairs) obtained after digestion with restriction enzymes. Only *Bgl* II showed differences between *A.m. caucasica* and the other two races tested

Eco RI	Hind III	Pst I	Bam HI	Acc I	Kpn I	Bgl II		
No difference in fragment pattern for all races						Apis mellifera		
						carnica	*caucasica*	*ligustica*
10.0	13.0	14.0	11.8	10.0	17.0	13.2	10.0	13.2
3.8	2.8	2.8	3.5	6.1	–	3.8	3.5	3.8
1.8	1.2	1.0	2.0	0.5	–	–	1.8	–
1.4	–	–	1.2	–	–	–	1.7	–
Total:								
17.0	17.0	17.8	18.5	16.6	17.0	17.0	17.0	17.0

But the homogenization of thoraces of adult bees was a major problem. The preparations of the mtDNA of flight muscle preparations were highly contaminated with genomic DNA. Larvae or early pupae gave much better results and showed a mtDNA band of adequate purity (fig. 1). In pupae, however, there was more RNA contamination than in the larval preparations. As the samples were treated with RNAase prior to the restriction digestion, this did not interfere with the analysis.

The results of seven different six-base enzyme cuts are shown in figure 2 and fragment sizes are given in the table. The size of the mtDNA of the honeybee is close to 17,000 base pairs (17,100±100) (fig. 2). Only the restriction pattern of *Bgl* II showed differences between the three races. For all other tested enzymes (*Eco* RI, *Hind* III, *Pst* I, *Bam* HI, *Acc* I, *Kpn* 1), no differences in the restriction pattern between the three races could be detected. The background smear in figure 2 is due to contaminating nuclear DNA. Nevertheless it was possible to identify mtDNA bands unambiguously. Control experiments showed that none of these bands derived from repeated sequences in nuclear DNA. The smear could be removed by further purification by ethidium bromide – cesium chloride gradient centrifugation; however this was not found necessary for interpretation of the gel patterns.

Various procedures are available for calculating the number of nucleotide substitutions per site differentiating two DNA sequences[17–19]. We used equating 20 of Nei and Li[17], treating the results of each enzyme separately to obtain a jackknife estimate of the number of nucleotide substitutions per site and its standard error of 0.0076±0.0089 for the comparison between *A.m. caucasica* and each of the other races.

Discussion. Our experiences showed that various tissues were not adequate for isolation of mtDNA from insects with hard endocuticles. In spite of the high concentration of mitochondria in flight muscle the yield is poor. This may be due to the elongated morphology of the flight muscle mitochondria which may render them prone to disruption during tissue homogenization. Larvae or pupae seem to be more suitable for the isolation of mitochondria. The soft tissue causes no problems during homogenization and the differential centrifugation step produces intact mitochondria well separated from nuclei. The size of approximately 17,000 base pairs for the circular DNA of honeybee mitochondria is well in the range of mtDNA sizes reported for other insects[20,21]. Our results also show that in Hymenoptera the technique of restriction enzyme analysis may reveal variability between conspecific populations. However the between-subspecies variability revealed in this study is very small. One explanation for the reduced variability in our study could be that the bees sampled did not originate from natural populations but from commercially bred stock. Therefore uncontrolled hybridization might have occurred before the lines were established and maintained by artificial insemination. The sample size was also fairly small. Looking at similarities in fragment pattern and differences in mtDNA sequence between races, we found less variation than has been reported for diploid populations in much smaller areas of geographical distribution[22–25]. In *Drosophila* larger mtDNA variabilities were found[21] even within a single matrilineal strain than was found in the present study for honeybees. A similar phenomenon has also been documented for cattle[26] where mtDNA mutations appeared within one maternal lineage.

Further experiments using 4-base and additional 6-base restriction enzymes to test natural populations should give more detailed evidence as to whether a reduced mtDNA variability is a general feature in male-haploid hymenoptera, especially in light of the large standard error for the delta estimate based on the seven enzymes used here.

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