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23. We thank J. Miller for help with DNA sequencing, K. Harris for technical assistance, R. Myers-Kniss for preparing the manuscript, and A. B. Zielinski for assistance and encouragement. This research was supported by research grants from the American Cancer Society (BC-86) and Abbott Laboratories, by the National Institutes of Health (CA-02897, and HD17103), by the National Science Foundation (INT-8313623), and by the Women's Board of the University of Chicago Cancer Research Foundation.

21 January 1986; accepted 31 January 1986

# An Ancient Developmental Induction: Heat-Shock Proteins Induced in Sporulation and Oogenesis

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**Every eukaryotic and prokaryotic organism tested to date synthesizes a small number of heat-shock proteins in response to heat and other forms of stress. A particular pattern of heat-shock gene expression was observed during ascospore development in *Saccharomyces*: heat-shock proteins hsp26 and hsp84 were strongly induced, whereas hsp70, the most highly conserved of these proteins, was neither induced nor inducible by heat shock. Instead, two proteins related to hsp70 were induced. A strikingly similar pattern of expression occurs during oogenesis in *Drosophila*, suggesting that it may be one of the earliest developmental pathways to evolve in eukaryotic cells.**

ALL ORGANISMS RESPOND TO MILD elevations in temperature by coordinately synthesizing a small set of heat-shock proteins. The exact number of proteins induced varies in different organisms, but in all cases proteins of approximately 84 and 70 kilodaltons (hsp84 and hsp70) are among the most prominent species. These proteins have been highly conserved in evolution. The hsp70 proteins of *Drosophila* and yeast have 72 percent amino acid identity (*1*) and their respective hsp84 proteins have 63 percent identity (*2, 3*). Most organisms also produce heat-shock proteins of 20 to 30 kD. *Drosophila* cells produce four closely related proteins of 28, 26, 23, and 22 kD. Cells of the yeast *Saccharomyces cerevisiae* produce only one prominent small protein with a molecular mass of 26 kD. These small heat-shock proteins have not been conserved to the same extent as hsp70 and hsp84, but nucleic acid sequence analysis has demonstrated homology among the proteins of insects, vertebrates, and nematodes (*4–5*). Furthermore, the small heat-shock proteins of *Drosophila*, yeast, and tomatoes form particles of highly conserved morphology (*6, 7*).

Although the specific functions of the heat-shock proteins are not yet known, some of them are expressed during oogenesis and pupation in *Drosophila* (*8–12*), suggesting that they play a role in normal development as well as in the response to stress. To investigate developmental regulation of the heat-shock genes in the yeast *S. cerevisiae*, we examined sporulating cells. Since these cells do not efficiently take up radiolabeled amino acids, gene expression was determined with DNA probes and antibodies. In the experiment represented in Fig. 1, diploid cells of the strain AP3 reached the tetranucleate stage 8 to 10 hours after transfer to nitrogen-deficient medium and sporulation was complete at 24 hours. Total cellular RNA's were isolated at various times during sporulation, electrophoretically separated, and analyzed by hybridization with cloned probes for the heat-shock genes.

Messenger RNA (mRNA) for hsp26 was induced early in sporulation, eventually reaching a concentration higher than that achieved during a 1-hour heat shock (Fig. 1a). Messenger RNA for hsp84 was also induced during sporulation (Fig. 1b). The timing of its accumulation was different from that of the hsp26 message. The maximum level of induction was comparable to that achieved with a 1-hour heat shock.

The hsp70 gene family in *Saccharomyces* contains two different classes of heat-inducible genes encoding 70-kD proteins. Transcripts from one class, represented here by clone YG100, are observed at low levels at 25°C and at much higher levels at 36°C. Transcripts of the other, represented by clone YG107, are observed only at temperatures above 38°C (*13*). Neither class was induced during sporulation (Fig. 1c). Moreover, as can be seen with longer exposures, as sporulation proceeded, the concentration of the YG100 message dropped below the basal level observed during normal vegetative growth.

We examined the expression of heat-shock RNA's in several *S. cerevisiae* strains of widely divergent genotypes. Messages for hsp26 and hsp84 are induced at different times in strains that sporulate at different rates, but they are always induced strongly. Neither class of hsp70 message was induced during sporulation in any strain. Thus, unlike the coordinate induction of these genes during heat shock, their induction during development is uncoupled; only a particular subset of heat-shock genes is induced.

This pattern of heat-shock gene expression is remarkably similar to one reported to occur during normal oogenesis in *Drosophila*. In adult females, RNA's for hsp26, hsp28, and hsp84 are induced in ovarian nurse cells and passed into the developing oocyte (*11*). As with meiosis in *S. cerevisiae*, this developmental induction differs from heat-shock induction in that mRNA for hsp70 does not accumulate. In fact, in late egg chambers and early embryos, hsp70 is not induced even with heat shock (*11*). This is significant, since, in virtually all other tissues, hsp70 is the protein most strongly induced by heat.

To determine whether hsp70 is heat-inducible during sporulation, we removed portions of a sporulating culture at various times during development and subjected them to heat shock at 39°C. RNA's from these cells were hybridized with probes for the two classes of hsp70 genes. Both were inducible during the early stages of sporulation; neither was inducible during the final stages of spore maturation (Fig. 2a). This change apparently occurred before general transcriptional inactivation of the spore genome, since an mRNA encoding a 21.5-kD sporulation-specific polypeptide accumulated after hsp70 became refractory to induction (Fig. 2b).

We translated RNA's from sporulating cells in vitro and found that the heat-shock messages they contained were fully translatable. However, the fact that these RNA's can be translated in vitro provides no infor-

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mation about their utilization in vivo. To analyze the expression of hsp26, we generated a polyclonal antibody specific for this protein. The antibody was raised by immunizing rabbits with electrophoretically purified 26-kD protein from heat-shocked cells. The antibody was highly specific. It reacted very strongly with proteins from wild-type cells that had been subjected to heat shock, but not with proteins from an isogenic strain in which the hsp26 gene was deleted (Fig. 3a) (*14*).

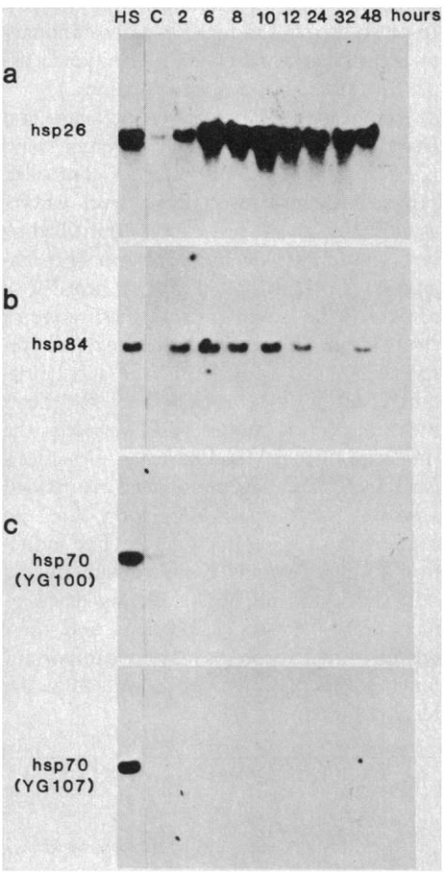


Fig. 1. Expression of heat-shock genes during sporulation. Total RNA's were extracted from diploid AP3 cells at various times after transfer to sporulation medium (*25*). For each time point, 2 μg of total RNA's were separated on 1.2 percent agarose gels containing 5 m*M* methylmercury hydroxide, transferred to nitrocellulose, and hybridized with $^{32}$P-labeled DNA probes for (a) hsp26, (b) hsp84, a 3′ probe specific for the heat-induced gene, and (c) hsp70 genes. Blots were hybridized as previously described (*24*) and washed in four changes of 0.2× standard saline citrate and 0.5 percent sodium dodecyl sulfate (SDS) at 42°C. Lane designations: HS, RNA's extracted from mid-logarthmic cells after a 1-hour incubation at 39°C; (c), RNA's extracted from mid-logarthmic cells grown at 30°C; 2 to 48 hours, RNA's extracted at various times after transfer to sporulation medium. The 32-hour time point is omitted from (b) and (c). The heat-shock lane was cut from another portion of the same blot. Hybridization of the blots to a ribosomal RNA probe demonstrated that the lanes were evenly loaded.

To examine the expression of hsp26 during sporulation, we extracted total cellular proteins by glass bead lysis in ethanol. The proteins were electrophoretically separated, transferred to nitrocellulose, and treated with the antibody. Accumulation of hsp26 was first detectable at 8 hours (Fig. 3b). This protein continued to accumulate during sporulation, becoming one of the major bands stainable with Coomassie blue on polyacrylamide gels of total cellular proteins. The band did not appear in proteins isolated from sporulating cells homozygous for the hsp26 gene deletion (*14*).

Studies in other systems have suggested that hsp70 is induced in particular cells at various stages of development (*15–18*). However, in those studies the heat-inducible 70-kD protein was not differentiated from related 70-kD proteins. Both the yeast and *Drosophila* genomes contain several genes (termed cognates) related to hsp70 (*19*). Some of the cognates are constitutively synthesized and not induced by heat (*1*). It is probable that a multigene family for hsp70 is present in the genomes of most eukaryotes.

We have shown that the yeast hsp70 genes are not expressed during sporulation, and others have shown that the *Drosophila* hsp70 genes are not induced during oogenesis (*11*). To investigate the expression of other members of this multigene family during development, we used monoclonal antibodies that discriminate between the heat-inducible and cognate proteins. These antibodies were obtained from a screen to select hybridomas against the major *Drosophila* heat-shock protein, hsp70. Most of the hybridomas generated produced antibodies that were highly specific for this protein (*20*). These antibodies (for example, 7Fb) reacted strongly with a single 70-kD polypeptide from heat-shocked cells and did not react with proteins from cells grown at normal temperatures (Fig. 4a).

Other hybridomas generated in this screen produced antibodies that recognized several members of the hsp70 gene family: hsp70, hsp68, and the cognate proteins (*21*). Antibody 7.10 reacted with two cognate protein bands from tissue culture (25°C) cells, ovaries, and gut tissues (Fig. 4b). In ovaries, one, if not both, of these proteins was induced relative to other tissues.

The 7.10 antibody recognizes an epitope that has been conserved on both the heat-inducible and cognate 70-kD proteins of most eukaryotic cells (*22*). In sporulating yeast cells, this antibody detected the accumulation of two protein bands, 71 and 70 kD, midway through spore development (Fig. 4c). Analysis of these samples on two-dimensional gels did not resolve them further (Fig. 4, d and e). Because we do not have an antibody specific for the yeast heat-inducible 70-kD proteins, we do not have rigorous proof that the proteins that accumulate during sporulation are different from the heat-inducible proteins. However, since the concentrations of the hsp70 mRNA's drop below the basal levels observed during vegetative growth, and since these messages become refractory to induction even with a heat shock, we consider this possibility unlikely.

Proteins related to hsp70 are induced in sporulating yeast cells and *Drosophila* ovaries. In addition, hsp26 and hsp84, which are induced during oogenesis in *Drosophila* (*11*), are also induced during sporulation in yeast. Further parallels are provided by the fact that hsp70 is not induced and is not inducible even with a heat shock.

The conservation of this pattern of gene expression in two such distantly related organisms implies that it may be a universal feature of gametogenesis. On first consideration, the *Xenopus* oocyte appears to be an exception. Hsp70 is inducible in these cells and hsp30 (the hsp26 homolog) does not accumulate (*23*). However, *Xenopus* oocytes are arrested at a late stage of meiosis, and it

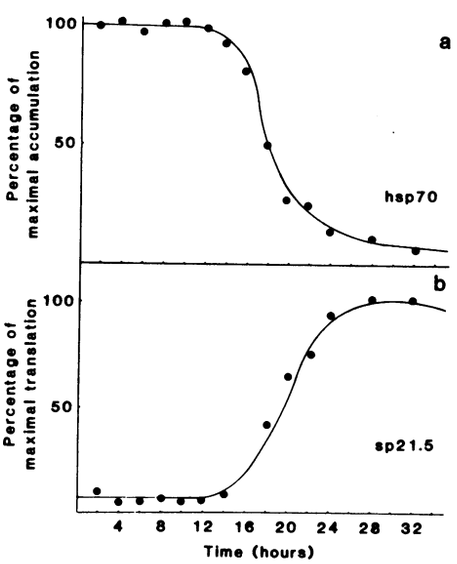


Fig. 2. (a) Inducibility of hsp70 during sporulation. At various times after transfer to sporulation medium, aliquots of cells were subjected to heat shock at 39°C for 1 hour. RNA's were extracted and analyzed as described in Fig. 1. Hybridization of the hsp70 probe (YG100) was normalized to that of the yeast ribosomal gene. (b) Accumulation of mRNA encoding the 21.5-kD sporulation-specific polypeptide. Sporulation RNA's were translated in vitro (*25*). Since RNA concentrations were below saturation for the lysate, the translation intensities of individual proteins reflected the relative abundance of their mRNA's. Accumulation of the 21.5-kD polypeptide (sp 21.5) was measured by laser densitometry of protein gel fluorograms.

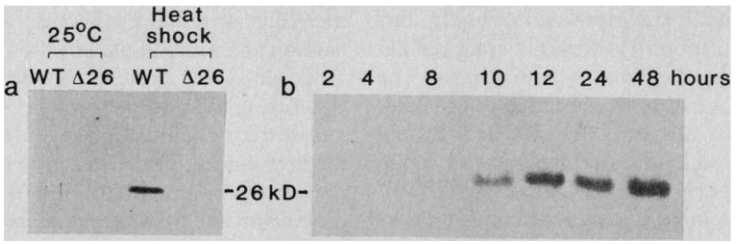


Fig. 3. (a) Specificity of yeast hsp26 antiserum. Cells were maintained at 25°C or subjected to heat shock for 1 hour at 39°C. Proteins were prepared from a wild-type (WT) yeast strain or from an isogenic strain in which the gene for hsp26 was deleted (Δ26). Electrophoretically separated proteins (*26*) were transferred to nitrocellulose and incubated with a 1:100 dilution of immune rabbit serum specific for yeast hsp26. The blots were developed with horseradish peroxidase–labeled goat antibody to rabbit immunoglobulin G (IgG) (Sigma) and 4-chloro-1-napthol (Bio-Rad) (*27*). (b) Accumulation of hsp26 during sporulation. After transfer to sporulation medium, aliquots of cells were removed at 2, 4, 8, 10, 12, 24, and 48 hours. Proteins were analyzed as described in the legend to (a). Rabbit serum specific for yeast hsp26 was prepared by injecting New Zealand White rabbits with proteins purified from the 26-kD region of an SDS–polyacrylamide gel. Comparison of heat-shock and control proteins on Coomassie blue–stained gels indicated that hsp26 was the only major band in this region.

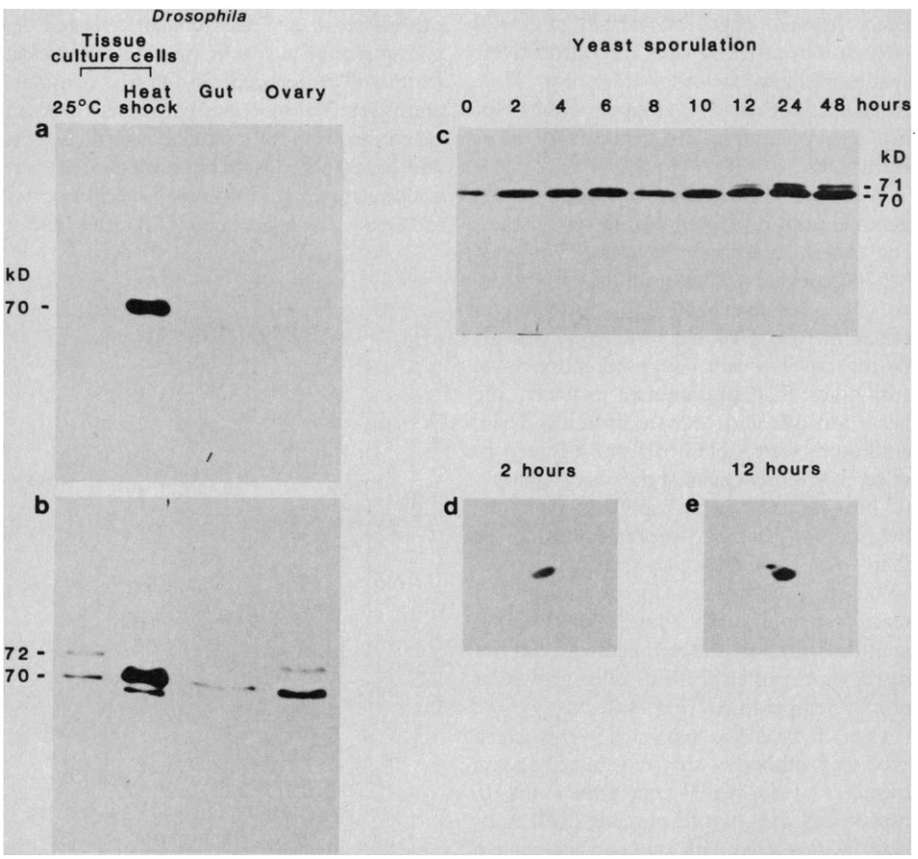


Fig. 4. Analysis of the hsp70 family during *Drosophila* and yeast development. (a) Equal amounts of protein from *Drosophila* tissue culture cells kept at 25°C or subjected to heat shock at 36.5°C and proteins from gut and ovary tissue of flies raised at 25°C were electrophoretically separated (*28*), transferred to nitrocellulose, and incubated with monoclonal antibody 7FB, which is specific for *Drosophila* hsp70. Blots were then washed, incubated with rabbit antibody to rat IgG, and developed with horseradish peroxidase–labeled goat antibody to rabbit IgG and 4-chloro-1-napthol. (b) Blot identical to that in (a) but reacted with monoclonal antibody 7.10, which recognizes several members of the hsp70 gene family in most eukaryotes. (c) After transfer to sporulation medium, aliquots of yeast cells were removed at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hours. Proteins were analyzed as in (b). (d and e) Proteins were isolated from sporulating yeast cells at 2 and 12 hours, separated on two-dimensional gels (*29*), transferred to nitrocellulose, and analyzed as in (b). *Drosophila melanogaster* (Oregon R strain) were grown in glass vials on standard banana medium at room temperature. Ovaries and gut tissue were dissected in ice-cold ethanol. Samples from 40 flies were homogenized by vortexing in 0.2 g of glass beads and 150 μl of 2× SDS sample buffer (*26*). Tissue culture samples were prepared as described by Velazquez *et al.* (*30*).

is only after fertilization that meiosis is completed. Recent research indicates that hsp70 induction is blocked immediately after fertilization (*4*). Since *Drosophila* hsp26 is part of a multigene family that is differentially expressed during development, it may be that a related, developmentally regulated protein will be found to accumulate in *Xenopus* oocytes as well.

It should be noted that developmental induction of heat-shock proteins is not restricted to gametogensis. In *Drosophila*, certain of the small heat-shock proteins are induced during puparium formation (*8–10*). In yeast, hsp26 is induced in early stationary phase cells and in *a/a* or α/α diploids exposed to nitrogen-deficient medium.

The heat-shock responses of yeast and *Drosophila* are regulated in different ways (*24*). In yeast the response is controlled primarily at the transcriptional level, whereas in *Drosophila* the response is controlled by both transcriptional and translational mechanisms. Furthermore, the yeast heat-shock response is transient over a broad range of temperatures, whereas the *Drosophila* response is not. Considering the substantial differences in the regulation of the heat-shock responses in yeast and *Drosophila*, the similarity of their developmental inductions is striking. Both organisms have maintained separate patterns of regulation for the expression of heat-shock genes. The induction of the proteins is coordinate during heat shock and uncoupled during development. The heat-shock proteins and their related cognate proteins may have important functions in development as well as in the response to stress.

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31. We thank E. Craig for YG100 and S. Johnson for preparing the manuscript. Supported by NSF grant PCM 8302611 by NIH grant GM 25874 to S.L., and by NIH predoctoral training grants GM 07138 to S.K. and J.R. and GM 07821 to L.P.

7 August 1985; accepted 12 November 1985

# Age-Dependent Changes in Proteins of *Drosophila melanogaster*

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**Several molecular theories of aging postulate that there are age-dependent changes in gene expression and that these changes contribute to the reduction in the viability of senescent cells. High-resolution, semiautomated, quantitative two-dimensional gel electrophoresis of many soluble proteins was used to test this hypothesis in *Drosophila*. Two-dimensional protein gel patterns were analyzed for each of three age groups of [$^{35}$S]methionine-labeled adult male *Drosophila melanogaster*, which, except for their spermatocytes, consist entirely of fixed postmitotic cells. Seven relatively abundant polypeptides expressed in middle-aged (28-day-old) flies were absent in both young (10-day-old) and old (44-day-old) flies. Quantitative analyses of an additional 100 polypeptides were carried out by computer-assisted microdensitometry of fluorograms of the gel preparations. These analyses revealed a significant age-related heterogeneity in the quantitative distribution of radiolabel in these proteins. The data indicate that the qualitative pattern of gene expression is identical in young and old flies, but that profound quantitative changes occur in the expression of proteins during the *Drosophila* life-span.**

ONE OF THE UNANSWERED QUESTIONS in gerontological research is whether significant alterations in gene expression occur with age. Thus, several current molecular theories of aging postulate that age-dependent changes occur in proteins and that such changes contribute to the reduction in the viability of senescent cells (*1–5*). Many aspects of gene expression have been evaluated in an attempt to clarify this issue (*6*). So far, studies aimed at detecting translational errors as a function of age have failed to provide any convincing evidence in favor of this hypothesis (*7, 8*). Observations in support of mutational theories of aging are also lacking (*9–11*). Neither amino acid sequence nor amino acid compositional alterations have been found to be age-dependent (*12*). In addition, previous studies involving the use of two-dimensional (2-D) gel electrophoresis to search for protein changes with senescence have generally been restricted to qualitative assessments, with the exception of several attempts at quantitative analyses of only a few polypeptides (*13, 14*).

Although the methodology used in these studies can provide significant information on variations in gene expression, such techniques are of limited value because alterations in the expression of proteins in old animals may be quantitative rather than qualitative. So far, no rigorous quantitative evaluation of polypeptides from animals of different ages, separated by high-resolution 2-D gel electrophoresis, has been reported. This is due in part to the inability of many laboratories to perform these types of analyses. In contrast to classical studies of protein turnover, semiautomated, quantitative 2-D gel electrophoresis provides an accurate measure of the magnitude of expression of a large array of individual polypeptides.

We used the imago of *Drosophila melanogaster* as a model of aging to look for qualitative changes in the expression of proteins by high-resolution 2-D gel electrophoresis (*15–17*). In spite of the sensitivity of this technique and the many proteins examined, the fluorograms did not provide evidence that there are charge differences in any single protein between young and old flies. Moreover, the analyses did not reveal changes in the molecular weight of any of the proteins from any of the age groups, that is, the qualitative pattern of gene expression by young and old *Drosophila* was identical. Therefore our data do not support theories that invoke molecular weight changes (protein cross-linking) or charge alterations (error catastrophe, mutations, or deamidation) as the mechanism for senescence (*2–5*).

Although one low molecular weight pro-

Table 1. Quantitative comparisons of all gels. Ten gels from each age group were analyzed. Two exposures of each gel were made to quantify both faint and dark spots without saturating the film. Standard deviations, calculated from the data in Fig. 2, were obtained from the protein spot mean for each protein within its group of gels. In a given comparison, the standard deviation is calculated from the distribution of differences of the means between the two groups over the 100 spots. The protein quantities are normalized, so each quantity is the percentage of the total protein quantity on the gel, which makes the mean of these differences 0. The greater than twofold differences indicate the number of proteins with at least twice as much or half the label of the corresponding protein in the group compared. Qualitative differences represent the number of proteins in which the intensity is at least 20 times greater than its counterpart in the comparative sample. The table also indicates the reproducibility of the method, as determined from data obtained as described in the legend to Fig. 2A.

| Compared | Quantitative data | | | | Qualitative data | |
|---|---|---|---|---|---|---|
| | Proteins compared | Correlation coefficient | Standard deviation ($\times 10^{-3}$) | Proteins more than twofold different | Proteins compared | Differences |
| Young versus young | 100 | 0.97 | 1.45 | 0 | >500 | 0 |
| Middle versus middle | 100 | 0.97 | 1.65 | 0 | >500 | 0 |
| Old versus old | 100 | 0.98 | 1.55 | 0 | >500 | 0 |
| Young versus middle | 100 | 0.87 | 3.55 | 2 | >500 | 7 |
| Young versus old | 100 | 0.77 | 5.60 | 10 | >500 | 0 |

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