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A Search for Principles of Disability using Experimental Impairment of *Drosophila melanogaster*

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Abstract

The results of life table experiments to determine the effects of artificial impairment (leg amputation) in 7,500 *D. melanogaster* adults revealed that the extent to which life expectancy was reduced in impaired individuals was conditional on: (1) leg location and number amputated—front leg had greatest impact and the number of legs amputated directly correlated with mortality impact; (2) age of amputation—the greatest relative reduction in remaining life expectancy occurred when young flies were impaired; (3) vial orientation—mortality in impaired flies was the least when vials held upside-down (most friendly environment) and the greatest when they were right-side up (least friendly environment); and (4) sex—male mortality was reduced more than female mortality in nearly all impairment treatments. These results were used to formulate a set of general principles of disability that would apply not only to humans but to all organisms.

INTRODUCTION

Although the potential for the loss of physical function as an outcome of aging or due to either deliberate or accidental trauma is part of the natural history of all organisms, the behavioral, ecological and evolutionary literature concerned with disability and impairment in both non-human species and humans living in pre-modern societies is as scattered and thin as it is unstructured and descriptive. For example, the results of studies of wear-and-tear in insects are considered largely in the context of either aging theory [1–3] or age-determination technology [4–7], and only rarely in the context of fitness [8–10]. The literature on impairments in non-human vertebrates includes studies concerned with healed fractures [11,12], degenerative joint disease [13–20], broken teeth and horns [21–23], and either diversionary [24–26], compensatory [27,28], or infirmity-elicited [29,30] behaviors. But there are no papers in the ecology, behavior and evolution literature that attempt to either generalize concepts or unify the field. Much of the literature concerned with injuries and disability in prehistoric [31–34]

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and indigenous [35] human societies focuses on the importance of sociality and provisioning in increasing post-trauma survival [13] but not on mortality implications or quality of life.

The paucity of information on the actuarial consequences of impairment in non-human animals is likely due, at least in part, to the absence of an experimental paradigm for manipulative investigations on the consequences of disability in model organisms. Therefore we initiated a study on *Drosophila melanogaster* to examine systematically the effects on survival and mortality of different types and levels of impairment, of the age of impairment onset, and of interactions and/or consequences of the environment. We use the results of these experiments to identify a set of general principles concerned with the effects of disability and impairment on mortality. Limb amputation was used to artificially impair flies since we believe that this is the only method available that not only provides a means for manipulating the severity and timing of impairment, but also eliminates the confounding effects of co-morbidities that are often present in naturally-occurring impairments.

METHODS

Conceptual Framework

We used the unified and standard language of the *International Classification of Functioning, Disability and Health* (36) as a baseline for the description of health and health-related states in fruit flies. A *pathology* thus refers to an abnormality that is detected and labeled as disease, injury or developmental condition, an *impairment* refers to a dysfunction and significant structural abnormality in a specific body system involving the loss or limitation of function on a long term or permanent basis, and a *disability* refers to an experienced difficulty doing activities in any domain of life (humans or animals) due to a health or physical problem. Therefore in the current study the *pathology* is the amputation (induced injury), the *impairment* is the loss of the limb(s), and the *disability* is the difficulty experienced by the fly resulting from the impairment. Whereas the disability is the proximate (immediate) source of increased risk, the pathology is the ultimate (underlying) source. We refer to limb (leg) amputation in fruit flies as an induced *impairment* involving different *levels* (number of legs surgically amputated), *configurations* (combination of leg amputations such as the removal of one front and one middle leg or of two middle legs), and *timing* (age at which impairment occurs), the consequences of which are influenced by the *environmental conditions* (vial orientation in the current study).

General

We studied the effects on the life table rates of leg impairments in three separate experiments involving approximately 7,500 *Drosophila melanogaster* adults (Oregon strain). Prior to impairments, flies were housed in four (30cm × 30cm × 30cm) fine-meshed cages with an ad lib food supply (standard diet consisting of 0.75% agar, 5.83% molasses, 10% cornmeal, 8.33% torula yeast, 1.67% ethanol, 0.33% methyl-phydroxybenzoate, 0.67% propionic acid) at 24° ± 3C, 65% relative humidity and a 12:12 L:D cycle, photo phase starting at 09:00. On the day preceding impairments, all flies were released from the cages to assure that only newly emerged flies were impaired the following day. Flies were captured, anesthetized using CO₂, and placed under a dissecting microscope. Fly sex was determined and legs were removed using a fine-tipped forceps and a dissecting needle. Flies were anesthetized for less than 5 minutes and monitored until recovery from the CO₂. Following the impairment, flies were housed individually in Polystyrene vials (diameter 2.5cm, height 10cm) sealed with a cotton ball containing standard fly food to maintain them throughout their lives. Flies were transferred to fresh vials if the food began to dry. Vials with treated flies were placed in cardboard boxes each containing 100 vials, organized in a Latin square design by treatment within the box with females and male cohorts maintained in separate boxes. Survival was checked daily at the same

time. Due to the 24 hour period between releasing the flies and starting impairments, some of the females mated prior to the impairment and laid eggs in their vials. These females were transferred into new vials to avoid confusion between the treated female and her offspring whenever larvae were detected.

Specific Experiments

The study involved the following three life table experiments. *Experiment #1: Impairment configurations.* Individual adults were randomly assigned to one of the following impairment (leg removal) treatments: one front, middle, or hind leg; two front, middle or hind legs; one front and one middle leg from the same side; one middle leg and one back leg from the same side; one middle leg and two back legs; and control (anesthetized and placed under the microscope without removing legs). *Experiment #2: Impairment timing.* On day 0 (eclosion), 7, 14 or 28 adults maintained individually in vials were anesthetized, placed under a microscope for amputation of one of their front legs, and returned to their vial where they were monitored until death. *Experiment #3: Impairment environment.* Newly-eclosed individual flies with either a single front, middle or hind leg amputated were randomly assigned to vials oriented either right-side up (normal), side-ways or upside-down and monitored daily for survival until death.

RESULTS

Impairment Level and Configuration

The longevity responses for both sexes of *D. melanogaster* to different amputation treatments are shown in Table 1 and Fig. 1. Several aspects of these results merit comment. First, life expectancy decreased with the number of legs amputated. For example, the average life expectancy for all treatments in which one, two and three legs were removed was 27.8, 15.9 and 9.5 days for females, respectively, and 23.5, 10.7 and 6.8 days for males, respectively. Additional or alternative multi-leg amputation treatments would change the overall averages for two- and three-leg amputation treatments. Second, the extent to which life expectancy was reduced was conditional upon the specific leg(s) amputated. Whereas amputation of the front leg either alone or in combination with other legs had the greatest impact on life expectancy relative to treatments that did not include the front leg, amputation of the middle leg(s) had the least impact. For example, amputation of a single front leg reduced life expectancy from over 43 days in females and 40 days in males to 20.6 and 13.4 days in females and males, respectively. In contrast, amputation of a single middle leg reduced life expectancy by only around 6 days in females (37.1 days) and 5 days in males (35.3 days). In other words, depending on sex a single front leg removed reduced life expectancy by 52 to 66% but a single middle leg removed reduced it by only 10 to 14%. Third, life expectancy reductions for treatments involving two legs depended on whether the two legs amputated were from the same side or from different sides. For example, life expectancy was reduced by around 55% for the three symmetrical (e.g. both front legs) amputation treatments but was reduced by over 70% for the two treatments involving amputation of two legs from the same side. More generally, flies experience near-catastrophic levels of mortality for several combinations or levels of impairment including the treatments in which both front legs (Fig. 1, middle panels) were amputated as well as the treatments in which two legs from one side and three legs were amputated (Fig. 1, bottom panels). Fourth, with the exception of the treatment involving the amputation of a single middle leg, life expectancy was reduced more in males than in females for the same treatment. For example, life expectancy in treatments involving single leg amputations in females and males was reduced by 35.6 and 40.4%, respectively, for two-leg amputations by 63.3 and 68.3%, respectively, and for the three-leg amputation treatment by 78.0 and 82.7%, respectively.

All treatments lead to significant impairments ($p\text{-values} \leq 10^{-3}$) except for the treatment involving the amputation of single middle leg, where there is evidence for impairment but it is not significant. The treatment involving amputation of both front legs and the three treatments involving asymmetrical amputations of 2 or 3 legs lead to the most critical impairments, as evidenced by hazard ratios ranging from 6.3 to 9.8 (Table 1). The treatments involving amputation of one front leg, amputation of both hind legs, amputation of three legs lead to significantly increased impairment for males as compared to females with hazard ratios for these comparisons ranging from 1.6 to 1.9. The shapes of the survival curves reveal the vulnerable periods in which the impairment seems to have crucial impact. The vulnerable periods differ according to treatment and between males and females, but much vulnerability is seen from eclosion through 20 days for many of the treatments and especially for males.

Comparisons of the hazard ratios given in Table 1 shed light of the observed combined risks, for example, of the observed joint risk of 2 legs being removed relative to the product of the single independent risks. For example, the product of the hazard ratio of one middle leg ($=1.318$) and two hind legs (3.748) yields the joint hazard ratio of these two treatments if the effects of the amputations were independent ($=4.940$). However, the observed hazard ratio for this specific treatment was 9.756 or a 2-fold greater hazard ratio than would be expected if the effects of each treatment (amputation levels) were independent. In short, the synergistic effects of two or more impairments may have multiplicative effects on the risk of dying.

Effect of Impairment Timing

The results of experiments designed to determine the effects of the age at which flies were artificially impaired on remaining life expectancy at eclosion, 7, 14 and 28 days are presented in Table 2. Impairment at 0 and 7 days is particularly devastating with hazard ratios of above 2.5. Impairment at 14 days is significantly associated with increased hazard ratio of about 1.8, while impairment at day 21 is not associated with significantly elevated risk. The gender differences are more subtle.

Statistical analysis using a model with no interaction terms reveals a strong gender effect and male flies are overall more severely affected by the impairment, with an overall hazard ratio of 1.3. However, inclusion of the interaction terms allows for a more detailed analysis in which case there is no overall significant gender effect. In this case we find that males are significantly more severely affected by impairment at day 0 (hazard ratio of around 2) and even more so by impairment at day 7 (hazard ratio of 3.7). This is of interest in light of the fact that for females, impairment at day 0 and at day 7 seems to have relatively similar effects. So the vulnerable periods as evidenced by the effect of impairment differ between male and female flies. A Cox proportional hazards model was fitted to the data, to relate the survival outcome during the first two weeks to the predictors, (a) type of treatment and (b) sex. Male flies suffered more under impairment overall (hazard ratio of 1.5). Removal of the front leg was worst (ratio = 3.6), while removal of middle and hind leg had a similar impact on hazard ratio (2.8 and 2.7, respectively).

Effect of Environment on Actuarial Impact of Impairment

We fitted a three-way ANOVA model to compare the mean mortality rate among each combination of sex, impairment treatments and vial orientations. Males have significant higher (5.7%) overall mortality than females for all the combination of impairment treatments and environments (Table 3). Removal of the front leg is more devastating than the removal of the middle or hind leg for both of males and females across all the impairment treatments. Different vial orientations had significant impact on mortality for both of males and females and for all impairment cohorts. Surprisingly the highest mortality of impaired flies was observed when they were maintained in vials in the upright (normal) position and lowest mortality when they

were in upside-down vials. The reason for the higher mortality of the impaired flies in the vials maintained in the upright, normal condition was due their heightened vulnerability for becoming stuck in the food at the bottom of the vial. This was not a problem for the impaired flies when the food was at the top of the vial because they would feed while standing on the glass side of the vial rather than stand or rest on the sticky diet itself. Depending on sex, flies missing the front leg experienced mortality 2- to 3-times greater for those maintained in vials held in the normal (upright) position relative to flies maintained in vials held sideways or upside-down. In contrast, mortality was lowest in vials held in the normal position for intact flies of both sexes. The up-right vial position made the effect of the removal of the front leg worse (hazard ratio for the combination was 2.6).

IDENTIFICATION OF GENERAL PRINCIPLES

The results of the experiments described in the current study involving different impairment levels, configurations, induction ages, and environmental conditions provide actuarial baselines and empirical context for identifying a preliminary set of general disability principles that both build on and complement general concepts derived from the human disability literature [37–45]. The main difference between studies on disability in humans and this study is that, whereas research on the consequences of human disability is concerned with reductions in quality of life resulting from naturally-acquired impairments with little emphasis on mortality consequences, our research on *D. melanogaster* was expressly concerned with changes in mortality and longevity resulting from artificially-impaired individuals. In other words, our studies focus on longevity and involve manipulative experiments whereas studies of humans focus on quality of life and are clinical, confounded, and descriptive.

We identify four principles concerning the mortality consequences of disability that we derive from our results and that we believe are general though not entirely universal. *Principle #1—impairments increase the risk of death.* Although there are a few exceptions to this principle in the literature such as the observation that removing the wings of house flies increased their longevity in the laboratory [8], our results involving limb amputation revealed that every impairment treatment lowered survival rates in both sexes. The general concept is that any disruption that compromises the integrity of an organism will reduce life expectancy. *Principle #2—multiple impairments have synergistic effects on mortality risk.* This principle underscores the importance of both cumulative morbidity and co-morbidity in that impairments which may have negligible effects by themselves (e.g. loss of a single middle leg). However, the effect of each impairment may be amplified when combined with other impairments as was revealed in the hazard ratio comparisons between single and multiple impairments. *Principle #3—the actuarial consequences of impairments are inextricably linked to the environment in which impaired individuals reside.* The primacy of the environment (vial orientation) on longevity is underscored by the results of the current study showing that *Drosophila* mortality differed was up to 3-fold less in a benign environment versus a ‘friendly’ environment for a specific impairment (e.g. loss of a single front leg). *Principle #4—the actuarial outcome of impairment is both sex and age-specific.* This principle is a simple extension of the observation that mortality all organisms including fruit flies [46] is age-and sex-specific. Because frailty varies by age and sex, it follows that the actuarial effect of any perturbation will differ by age and sex. This is underscored by the findings in the current study.

GENERAL DISCUSSION

Inasmuch as chronic disability is considered by the biomedical and public health establishments as the single most important issue in health care confronting developed countries such as the U.S. in the 21st century [37–39,47–49], it follows that new approaches are needed that are designed to shed new light on disability processes and to strengthen conceptual foundations

for disability research [43,44,50,51]. We believe that the research on impairment in model organisms is important for at least two reasons. *First*, our manipulative approach to understanding the effects of impairment on mortality provides a general experimental framework for studies designed to shed important light on other aspects of disabilities, particularly those that are relevant to humans such as the identification of impairment sequences, the relationship of frailty, morbidity, and mortality, and the dynamics of co-morbidity and sex-impairment differentials. For example, the current models involving impairments and disability described in the World Health Organization's international classification of function, disability and health [36] are based on sequences of qualitatively-different 'events' including the 'progression' from pathology (disease; abnormality; injury) to impairment (dysfunctions in body systems) to disability (experienced difficulty) to handicap (disadvantage relative to others)] rather than sequences of different stages or levels of impairments.

Second, impairment research on non-human species will provide context and scope for studies in ecology, evolution and behavior ranging from population ecology and predator-prey interactions to community ecology and sociobiology. Integrating disability concepts into population biology will encourage ecologists to consider distinguishing between the underlying causes of death (e.g. disabilities) and the immediate causes of death (predators; parasites), evolutionary biologists to factors endpoints other than death into fitness concepts, and behaviorists to expand their paradigm to include studies of how behavior changes with age, how behavior of older individuals differs from the behavior of young ones [52], and the how the acquisition of disabilities and behavior are mutually affecting.

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Fig 1.

Sex-specific survival of *D. melanogaster* subject to different impairment treatments: top panels—single-leg impairments; middle panels—two-leg impairments (complementary pairs); bottom panels—complex impairments. Survival of control (intact) cohorts (bold line) shown in top panels are included in middle and bottom panels for reference.

Sex-specific life expectancy (days) and hazard ratios by impairment treatment (plus control). Percent reduction refers to the reduction in the life expectancy of the treatment cohort relative to the life expectancy of the female (=39.4 days) and male (=43.2 days) cohorts[†]. Treatment refers to which leg or legs were amputated in the cohort.

Life expectancy was reduced by an average of 38.0, 65.8 and 80.4% in response to 1, 2 and 3 legs removed, respectively. For all treatments, life expectancy was reduced by 59.0% for females and

3 Opposite sides

Table 2

Remaining sex-specific life expectancy (e_x in days) for *D. melanogaster* adults for experiment #2 in which a single front leg was removed at age $x=0, 7, 14$ or 28 .

x	Control		x=0		x=7		x=14		x=28	
	Control	SD	e_x	SD	e_x	SD	e_x	SD	e_x	SD
Females										
0	38.0	14.1	19.4	12.6	18.8	13.4	26	14	35.8	10.2
7	32.0	13.0	13.8	12.2	11.9	13.4	19	14	28.8	10.2
14	26.2	12.0	13.9	11.1	13.5	13.7	12.3	13.9	21.8	10.2
28	15.4	10.4	11.8	8.4	18.7	17.1	14	11.2	7.8	10.2
Males										
0	41.3	14.8	14.2	10.1	10.3	6.7	25	16.8	38.1	13.8
7	35.2	13.8	9.4	10.2	3.3	6.7	18	16.8	31.1	13.8
14	28.8	13.2	10.6	11.0	13.4	15.4	11.0	16.8	24.1	13.8
28	18.3	11.0	16.5	11.0	16.3	11.8	26.1	13.0	10.3	13.8

Table 3

Percent mortality for *D. melanogaster* adults for experiment #3 in which a single front, middle or hind leg was removed and individuals maintained in vials that were oriented right-side up (i.e. normal with food on bottom), side ways, or upside-down (i.e. food on top).

Sex/Trt	Normal		Sideways		Upside-down		Means	
	mean	SD	mean	SD	mean	SD	mean	SD
Female								
Control	4.0%	0.1969	8.0%	0.2727	9.0%	0.2876	7.0%	0.3000
Front	48.0	0.5021	31.7	0.4677	14.9	0.3574	31.4	0.5000
Middle	20.0	0.4020	23.0	0.4230	15.0	0.3589	19.3	0.4000
Hind	29.0	0.4560	19.0	0.3943	12.8	0.3355	20.4	0.4000
Male								
Control	7.0%	0.2564	7.0%	0.2564	12.0%	0.3266	8.7%	0.2800
Front	54.0	0.5009	43.9	0.4986	28.3	0.4527	42.2	0.5000
Middle	20.0	0.4020	24.0	0.4292	16.0	0.3685	20.0	0.4000
Hind	36.7	0.4846	29.8	0.4598	24.4	0.4322	30.3	0.5000