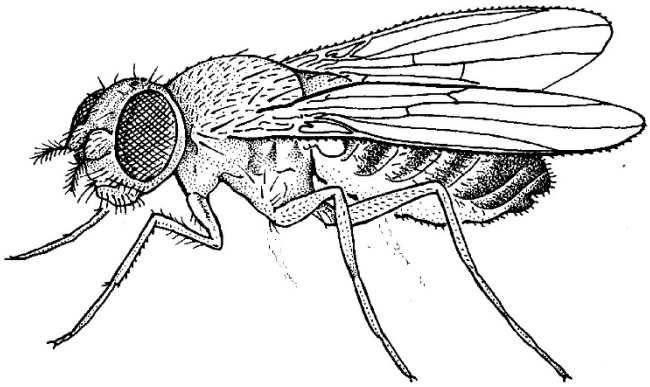
Drosophila Information Service



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Preface

Drosophila Information Service (often called "DIS" by those in the field) was first printed in March, 1934. For those first issues, material contributed by *Drosophila* workers was arranged by C.B. Bridges and M. Demerec. As noted in its preface, which is reprinted in Dros. Inf. Serv. 75 (1994), Drosophila Information Service was undertaken because, "An appreciable share of credit for the fine accomplishments in *Drosophila* genetics is due to the broadmindedness of the original *Drosophila* workers who established the policy of a free exchange of material and information among all actively interested in *Drosophila* research. This policy has proved to be a great stimulus for the use of *Drosophila* material in genetic research and is directly responsible for many important contributions." Since that first issue, DIS has continued to promote open communication.

Except for the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the annual volumes, all issues are now freely-accessible from our web site: www.ou.edu/journals/dis. For rare early issues that exist as aging typed or mimeographed copies, some notes and announcements have not yet been fully brought on line. But we intend to fill in those gaps for historical purposes in the future. Printed copies of recent annual issues can be obtained from www.lulu.com.

We continue to encourage all researchers to consider submitting articles that use *Drosophila* for teaching as well as articles that report new techniques, research results, and interesting new mutations. In the interests of honoring the long-standing philosophy of open exchange of ideas, we sometimes accept articles that have unusual or limited perspectives. We thank the many contributors from around the world who sent material for this issue, and we invite your submissions for future annual issues as well as any suggestions you have for maintaining this as a useful *Drosophila* research community resource. Unfortunately, some computer malfunctions and other factors delayed the printing of this volume, but all research articles were accepted for publication on or before 31 December 2023.

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List of Contributions

General Announcements

Research and Teaching Notes

Other Reports

Research and Teaching Notes

Occurrence of *Zaprionus tuberculatus* on Southeastern Brazil coastal plain, in Rio de Janeiro.

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Zaprionus tuberculatus Malloch 1932 is an African drosophilid included in the *inermis* species group. This fly expanded its geographic range to Eastern and Western Mediterranean lands about a decade ago (Patlar *et al*., 2012; Raspi *et al*., 2014), and more recently it was detected for the first time in Americas (Cavalcanti *et al*., 2022). Moreover, since this species is a potential agricultural pest – as observed for the invasive *Zaprionus indianus*, already established in South, Central, and North America – a close monitoring of its presence in neotropical areas is in order.

Z. tuberculatus first record in the American continent was obtained in the Brazilian central highlands. Firstly, it was detected in natural conservation units around Brasilia, in January 2020. About one year later its presence was detected in urban parks, in Brasilia (Cavalcanti *et al*., 2022). Here, we communicate its identification at two localities on the coast of the state of Rio de Janeiro, Southeastern Brazil. Moreover, in both areas we observed *Z. tuberculatus* in lower abundance than *Z. indianus*.

Our first record of *Z. tuberculatus* was obtained from blackberry fruits collected in March 2022 from an orchard at Araruama (22º 52’ 22’’ S; 42º 20’ 35’’ W), a beach town located at approximately 120 km eastwards from Rio de Janeiro. We collected these fruits and brought them to our laboratory as a methodological test of procedures for the study of fly predation on fruit plants. Each blackberry was kept on a cotton cushion, inside a plastic cup covered with a net tissue (2 mm mesh), at 22°C. From a total of 25 collected fruits, four yielded nine *Zaprionus* flies. Of these, eight were *Z. indianus* and one was a female *Z. tuberculatus*. This latter species was recognized due to the large tubercle on the forefemur and its adjacent cuticular expansion. Additionally, we observed that living individuals from these species can be easily distinguished by thorax tegument color. *Z. tuberculatus* displays a gray-brownish tone, darker than *Z. indianus* color.

In September 2022, two baits of banana and yeast were set up in that same orchard, and the flies attracted to the site were collected with a net. A total of 37 *Zaprionus* flies were collected, displaying the following proportion: 29 *Z. indianus* (17 males, 12 females) to 8 *Z. tuberculatus* (4 males, 4 females). We also collected blackberries, but no flies emerged from them. One month later, October 2022, we made one more blackberry collect and, again, no flies emerged from the collected fruits. It should be mentioned that all blackberry collections were obtained from a single plant.

Finally, in the third week of November 2022, we set up a trap – with banana and yeast bait – in a garden inside Federal University of Rio de Janeiro (UFRJ) campus, in Rio de Janeiro (22°50’20” S; 43°13’40” W). After two days all flies (most drosophilids) were collected and the trap was reset for four more days, allowing us to obtain a second collect. The first obtained sample contained four male and three female *Z. tuberculatus*, and no *Z. indianus* flies. The second contained 6 males and 5 females for *Z. indianus*, plus two males and one female *Z. tuberculatus*.

The main issue in this communication is the regular recording of *Z. tuberculatus* on Southeastern Brazil coastal plains, side by side with *Z. indianus*. In spite of the small sample size, and the result obtained from one of the trap samples obtained in Rio de Janeiro, our data suggest that the more recent invader species (*Z. tuberculatus*) is less common than *Z. indianus*. Besides that, our observations confirm the need of checking the ability of *Zaprionu*s flies to successfully lay eggs on small thin-skinned ripening fruits (Bernardi *et al*., 2017; Pfeiffer *et al*., 2019), which includes the activity of the most recent invader.

Acknowledgments: We are grateful to SISBIO for the authorization to collect Drosophilidae (85366-1) and to CNPq for financial support (408479/2021-3).

References: Bernardi, D., F. Andreazza, M. Botton, C.A. Baronio, and D.E. Nava 2017, Neotrop. Entomol. 46: 1-7; Cavalcanti, F.A.G.S., L.B. Ribeiro, G. Marins, G.S.S.S. Tonelli, S.N. Báo, A. Yassin, and R. Tidon 2022, Ann. Entomol. Soc. Am. 115 (3): 267-274; Patlar, B., B. Koc, M. Yilmaz, and E.D. Ozsoy 2012, Dros. Inf. Serv. 95: 94-96; Pfeiffer, D.G., M.E. Shrader, J.C.E. Wahls, B.N. Willbrand, I. Sandun, K. van der Linde, C.A. Laub, R.S. Mays, and E.R. Day 2019, J. Integr. Pest Manag. 10 (1): 1-8; Raspi, A., A. Grassi, and G. Benelli 2014, Bull. Insect. 67 (1): 157-160.

The effects of Bisphenol A (BPA) on *Drosophila melanogaster* lifespan.

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Abstract

This study aimed to determine the effects of bisphenol A (BPA), an endocrine disrupting chemical that is frequently used around the world, on the lifespan of *Drosophila melanogaster*. 0.1 mg/L, 1 mg/L, and 10 mg/L BPA were applied to the larvae of *D. melanogaster,* and the lifespans of adult females and males developed from these larvae were determined. 1 mg/L and 10 mg/L BPA shortened the lifespan of treated female fruit flies compared to untreated controls ($p < 0.05$), while 0.1 mg/L, 1 mg/L BPA reduced the lifespan of male fruit flies ($p < 0.05$). Keywords: Bisphenol A (BPA), endocrine disruptors, *Drosophila melanogaster*, lifespan.

Introduction

Endocrine disruptors (EDCs) are chemicals or substances that can interfere with the normal functioning of the endocrine system in humans and animals. EDCs can mimic, block, or alter the action of hormones in the body, leading to a disruption in the normal hormonal balance. They can bind to hormone receptors, interfere with hormone production, metabolism, or transport, or disrupt the signaling pathways involved in hormone regulation. These substances can be naturally occurring or man-made, and they can be found in a wide range of products and sources, including pesticides, plastics, personal care products, industrial chemicals, pharmaceuticals, and certain food additives. Some common examples of endocrine disruptors include bisphenol A (BPA), phthalates, polychlorinated biphenyls (PCBs), dioxins, pesticides (such as organochlorines and organophosphates), and certain flame retardants (Casals-Casas and Desvergne, 2011).

Bisphenol A (BPA; 4,4'-dihydroxy-2,2-diphenylpropane) is a chemical compound that has been widely used in the production of certain types of plastics and epoxy resins. It has been used in the manufacturing of various consumer products, including food and drink containers, water bottles, thermal paper (such as receipts), medical devices, and dental sealants. Its purpose is to provide strength and durability to plastics and to create a protective lining in cans and containers to prevent corrosion and contamination (Rochester, 2013; Seachrist *et al*., 2016). Recent studies have indeed shown that BPA can leach out of certain products into the surrounding environment or food and beverages they come into contact with. BPA has also been reported to be considered a xenoestrogen that has been shown to exhibit estrogenic activity, meaning it can bind to estrogen receptors in the body and mimic the effects of natural estrogen. This can disrupt the normal hormonal balance and signaling pathways regulated by estrogen (Rubin, 2011). The effects of BPA have been investigated in various vertebrate species, including humans, and have been found to act as a potent endocrine disruptor at environmentally relevant levels. Both agonistic and antagonistic activities of BPA have been reported in invertebrates (Hutchinson, 2002; Crain *et al*., 2007). Also, it was reported that BPA is a xenoestrogen that potentially can have adverse effects on *Drosophila melanogaster* (Atli and Unlu, 2012; Atli, 2013; Weiner *et al*., 2014; Begum *et al*., 2020; Senthil Kumar, 2022).

Aging is an inevitable process determined by a combination of genetic and environmental factors. It is known that external factors such as temperature, nutrition, radiation, and population density are effective in aging (Çakır and Bozcuk, 2000; He and Jasper, 2014). The effect of environmental toxic substances on aging is one of the research topics. The health risks of BPA, an environmental toxic substance found in the products we frequently use in our daily lives, are a growing concern. Identifying the effects of exposure to BPA on the aging process is greatly helpful in understanding the long-term effects of this substance on living things, especially humans. However, many of BPA's effects on the aging process are largely unknown, due to the technical difficulties of studying aging in mammals (Tan *et al*., 2015). For this reason, model organisms such as *D. melanogaster* can be used to determine the effects of BPA on aging.

D. melanogaster is an excellent model for studying the aging process. It has been also shown to be a good model for monitoring environmental toxicants especially due to its short developmental cycle, convenient husbandry, and facile genetics. The present study aimed to investigate the effects of BPA exposure on the aging process in *D. melanogaster*.

Materials and Methods

The organism, environmental conditions

The wild-type Oregon strain of *D. melanogaster* was reared on a standard cornmeal *Drosophila* medium at 25±1°C with relative humidity of 50-60% and in 12 hrs light, 12 hrs dark periods. The standard cornmeal contains corn flour, sugar, yeast, agar, and propionic acid.

The solutions of BPA were prepared from solid compounds (Sigma-Aldrich; Steinheim, Germany). A known amount of BPA was diluted in 1 mL acetone and it was filled to 1 L with 5% sucrose (Merck; Darmstadt, Germany) solution to prepare stock solutions. An acetone control group was used in the experiment, and all experimental groups except the control group were made up to the same concentration of acetone which was 1 mL per liter.

BPA exposures and lifespan experiments

Virgin Oregon females and males of the same age were crossed in culture bottles. Individuals were then removed from the culture bottles after 8 hours. 72±4 hrs later, the third instar larvae were collected. The larvae were exposed to 0.1 mg/L, 1 mg/L, and 10 mg/L BPA for six hours in glass tubes (2.5 × 7.5 cm) containing drying papers that had absorbed stock solutions. Dose selection was based on results from our previous studies.

After the exposures, BPA-exposed and non-exposed (control groups) larvae were placed in 250 mL glass bottles that contained a standard *Drosophila* medium. All experimental groups were taken into the culture room and the virgin females and males from these larvae were collected. Experimental groups were formed with 110 females and 110 males from the collected adult individuals. Male and female individuals were placed in groups of 10 in tubes containing standard medium. During the transfer to fresh medium twice a week, dead flies (if any) were recorded and removed from the environment. Individuals who escaped during the transfer were excluded from the total number. Transfers continued until the last individual died.

Statistical Analysis

The statistical analysis of the results was carried out using the SPSS 15.0 program. The lifespan of males and females was calculated with the ANOVA test. For all statistical analyses, the criteria for significance was $p < 0.05$.

Table 1. Average lifespan of females of *D. melanogaster* non-exposed (control and acetone control) and exposed to BPA.

Group No.	Groups	No. of females	Mean lifespan (Day) ± SE	SD	Significant differences in the means
1	Control	103	74.77 ± 1.13	11.52	
2	A. Control	105	75.12 ± 1.18	12.06	2-4*
3	0.1 mg/L	103	75.19 ± 1.18	12.03	2-5*
4	1 mg/L	103	71.58 ± 1.06	10.72	
5	10 mg/L	106	71.58 ± 1.07	10.98	

SE: Standard error, SD: Standard deviation, *p<0.05

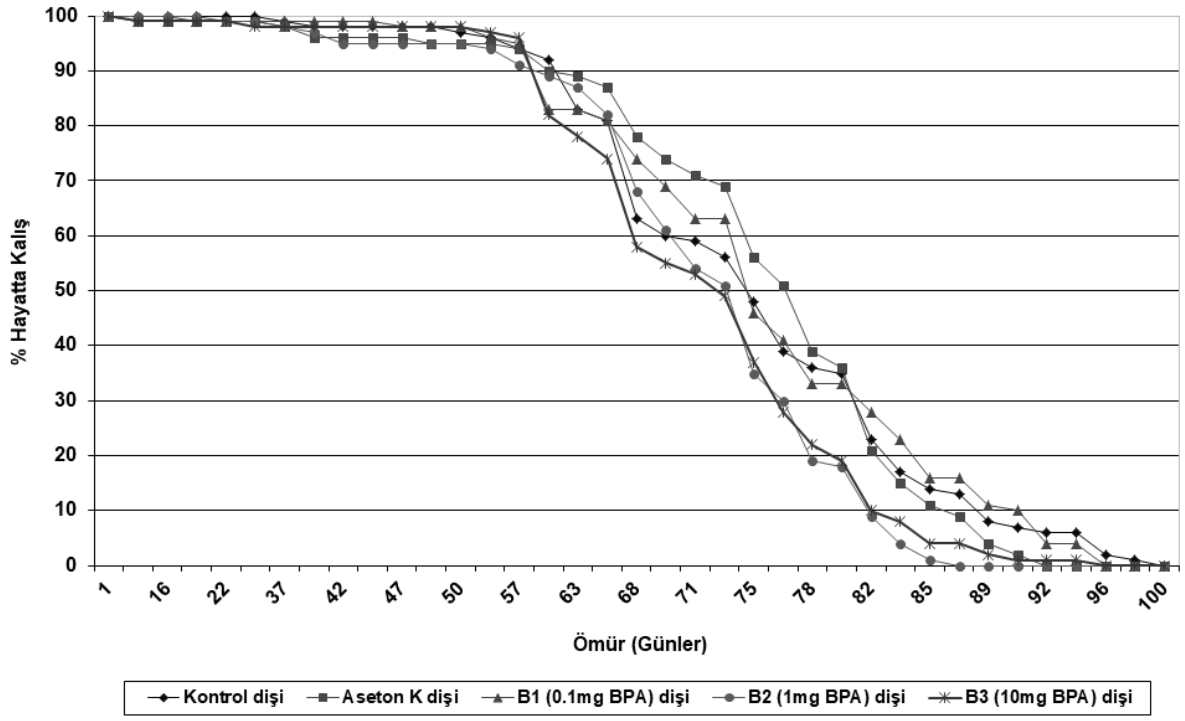

Figure 1. Survival curves of females of *D. melanogaster* non-exposed (control and acetone control) and exposed to BPA.

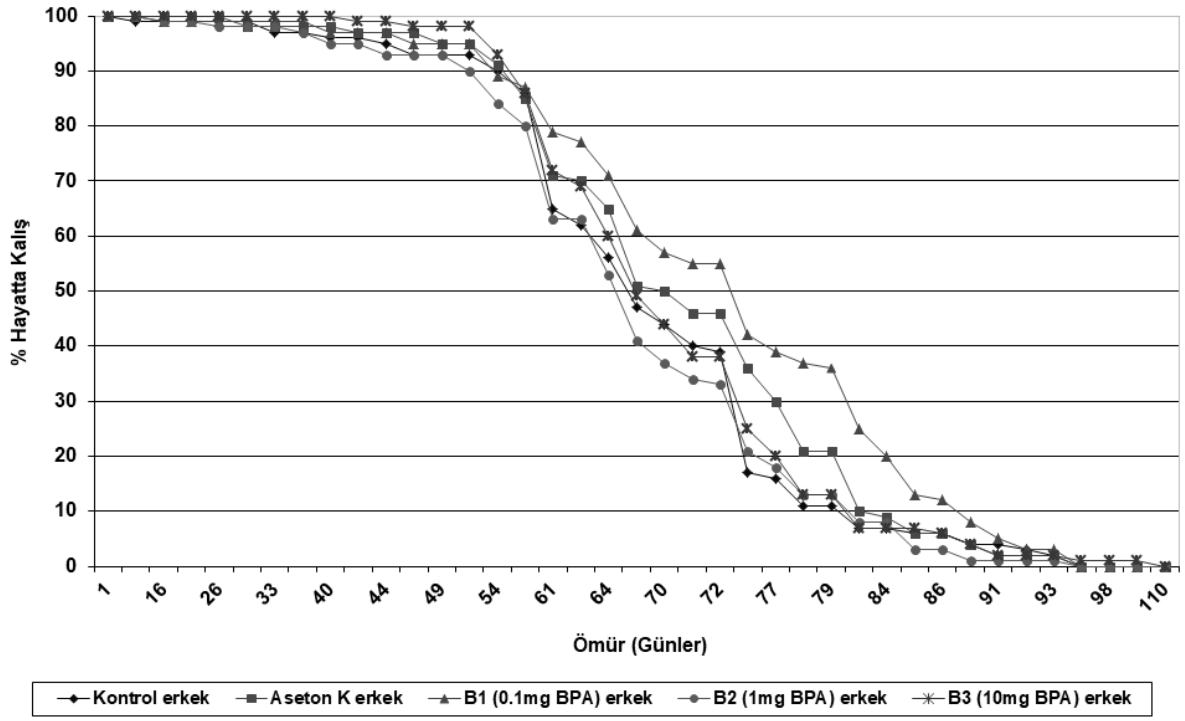

Figure 2. Survival curves of males of *D. melanogaster* non-exposed (control and acetone control) and exposed to BPA.

Table 2. The average lifespan of males of *D. melanogaster* non-exposed (control and acetone control) and exposed to BPA.

Group No.	Groups	No. of females	Mean lifespan (Day) ± SE	SD	Significant differences in the means
1	Control	97	68.20 ± 1.20	11.77	
2	A. Control	105	70.06 ± 1.18	12.11	1-3*
3	0.1 mg/L	106	72.73 ± 1.33	13.66	2-3*
4	1 mg/L	103	66.47 ± 1.28	13.03	
5	10 mg/L	96	69.51 ± 1.10	10.79	

SE: Standard error, SD: Standard deviation, *p<0.05

Results

Effect of the BPA on the female lifespan

Our results showed that the treatment of wild-type Oregon fruit flies with 1 and 10 mg/L concentrations of BPA has shortened the lifespan of female fruit flies when compared to acetone control ($p < 0.05$) (Table 1, Figure 1).

Effect of the BPA on the male lifespan

When evaluating the longevity of male flies (Table 2, Figure 2), it was observed that 0.1 mg/L BPA increased the life span of the exposed flies compared to the control group ($p < 0.05$).

Discussion

Bisphenol A (BPA) is a chemical compound that has been widely used in the manufacturing of various plastic products and epoxy resins since the 1960s. BPA can be found in polycarbonate plastics. It is also used in the manufacture of food storage and beverage containers, in epoxy resins, and for coating the interior surfaces of metal products such as food cans, bottle caps, and water supply lines (Cunha and Fernandes, 2020). However, concerns have arisen about the potential health risks associated with BPA exposure. Researches have suggested that BPA can leach from these products, especially when they are exposed to high temperatures or acidic substances, such as when heating food in plastic containers or drinking hot beverages from plastic cups. BPA is suspected to be an endocrine disruptor, meaning it can interfere with the body's hormonal system (Manzoor, *et al.*, 2022). In the current study, BPA exposures caused different effects on the lifespan of adults. While 1 mg/L and 10 mg/L BPA shortened the lifespan of females, 0.1 mg/L BPA increased the lifespan of males compared to control groups.

The number of studies investigating the effects of BPA on longevity is limited. Some research has indicated that exposure to BPA might lead to a shortened lifespan in certain invertebrate species. For example, a study conducted on nematode (*Caenorhabditis elegans*) suggested that exposure to BPA was associated with reduced lifespan (Tan *et al*., 2015). In a similar study conducted with *C. elegans*, it was determined that 1 mM BPA exposure shortened lifespan. At the same time, BPA exposure also led to developmental delay, decreased body growth, decreased reproduction, and abnormal tissue morphology in *C. elegans* (Hyun *et al*., 2021). In a study conducted by Park *et al*. (2018) with marine rotifer *Brachionus koreanus*, it was found that BPA exposure significantly shortened lifespan.

It was determined that BPA applied in the current study affected the longevity of males and females differently. The reason for this difference may be that hormones and antioxidant enzymes work differently in males and females. There are studies in the literature that support these results. In a study by Musachio *et al*. (2022), female and male *D. melanogaster* were separately exposed to BPA for seven days. It was determined that 0.5 and 1 mM BPA shortened the lifespan of males, while 0.25, 0.5, and 1 mM BPA shortened the lifespan of females. It was also found that the toxicological effects seen with BPA exposures were different between genders and that females were more susceptible to oxidative cell damage when exposed to BPA. Liu

et al. (2014) concluded that exposure to endocrine disruptors reduced the lifespan of females and did not change the lifespan of male flies. The greater reduction in lifespan in female flies than in males after exposure to endocrine disruptors has been attributed to decreased homeostasis of the ecdysone hormone.

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References: Atli, E., 2013, Bull. Environ. Contam. Toxicol. 91: 433–437; Atlı, E., and H. Ünlü 2012, Hacettepe Journal of Biology and Chemistry 40(1): 61-68; Begum, M.P. Paul, D. Das, and S. Ghosh 2020, Toxicol. Environ. Health Sci. 12: 237–246; Casals-Casas, C., and B. Desvergne 2011, Annual Review of Physiology 73(1): 135-162; Crain, D.A., M. Eriksen, T. Iguchi, S. Jobling, H. Laufer, G.A. LeBlanc, and L.J. Guillette 2007, Reproductive Toxicology 24(2): 225-239; Cunha, S.C., and J.O. Fernandes 2020, *In* Handbooks in Separation Science, Liquid-Phase Extraction, Elsevier, 643-665; He, Y., and H. Jasper 2014, Methods 68(1): 129-133; Hutchinson, T.H., 2002, Toxicol. Letters 131: 75; Hyun, M., L. Rathor, H.J. Kim, T. McElroy, K.H. Hwang, S. Wohlgemuth, S. Curry, R. Xiao, C. Leeuwenburgh, J.D. Heo, and S.M. Han 2021, Toxicology 461: 152924; Liu, T., Y. Li, X. Zhao, M. Zhang, and W. Gu 2014, J. Insect Physiol. 71: 1–7; Manzoor, M.F., T. Tariq, B. Fatima, A. Sahar, F. Tariq, S. Munir, S. Khan, M.M.A. Nawaz Ranjha, A. Sameen, X.A. Zeng, and S.A. Ibrahim 2022, Frontiers in Nutrition 9: 1047827; Musachio, E.A.S., M.R. Poetini, D.E. Janner, L.B. Meichtry, K.H. Poleto, E.J. Fernandes, G.P. Guerra, and M. Prigol 2022, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 257: 109329; Park, J.C., M.C. Lee, D.S. Yoon, J. Han, M. Kim, U.K. Hwang, J.H. Jung, and J.S. Lee 2018, Aquat. Toxicol. 199: 21–29; Rochester, J.R., 2013, Reproductive Toxicology 42: 132-155; Rubin, B.S. 2011, The Journal of Steroid Biochemistry and Molecular Biology 127(1–2): 27-34; Seachrist, D.D., K.W. Bonk, S.M. Ho, G.S. Prins, A.M. Soto, and R.A. Keri 2016, Reproductive Toxicology 59: 167-182; Senthil Kumar, S., A. Swaminathan, M.M. Abdel-Daim, and S.S. Mohideen 2022, Mol. Biol. Rep. 49: 10703–10713; Tan L., S., Wang, Y. Wang, M. He, and D. Liu 2015, Toxicol Lett. 235(2):75-83; Weiner, A.K., A. Ramirez, T. Zintel, R.W. Rose, E. Wolff, A.L. Parker, K. Bennett, K. Johndreau, C. Rachfalski, J. Zhou, and S.T. Smith 2014, Ecotoxicology and Environmental Safety 101: 7-13.

Courtship behavior in the *Drosophila bipectinate* complex.

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Introduction

The *Drosophila bipectinata* complex is part of the *ananassae* subgroup of the subgenus Sophophora. It comprises eight species, including the subspecies *D. bipectinate bipectinata*, *D. bipectinata szentivani*, *D. bipectinata pacificiae, D. parabipectinata*, *D. malerkotliana malerkotliana*, *D. malerkotliana pallens*, *D. pseudoananassae pseudoananassae*, and *D. pseudoananasssae nigrens* (Bock, 1971, Matsuda *et al.*, 2005). It is widely distributed and includes Africa, the Indian Ocean islands, Southeast Asia, Northern Australia, the South Pacific Islands, and Brazil (Bock, 1971, 1980; Bock and Wheeler, 1972; Tomimura *et al*., 2005; Val and Sene, 1980). Furthermore, Aotsuka and Tobari (1983) reported that populations of the *D. bipectinata* complex collected from a wide area exhibited geographic divergence in isozyme polymorphisms. Additionally, they reported divergence among members of the complex in their morphological characteristics and sexual isolation. However, the degree of divergence among species for each of these characteristics was not consistently parallel, especially among closely related populations. Kopp and Barmina (2005) reported that this complex comprises four closely related, largely sympatric species. They also showed that *D. bipectinata* and *D. parabipectinata* are the two most closely related species, along with *D. malerkotliana*, they form a monophyletic clade to which *D. pseudoananassae* is a relatively distant outgroup based on the sequences of one mitochondrial and six nuclear loci. Based on their data, the estimated number of species in the complex was extremely low, and we estimate that these species only diverged 283,000–385,000 years ago.

Additionally, using the courtship behavior of the four species of the *D. bipectinata* complex, Hegde and Krishnamurthy (1979) reported that the species relationships inferred from behavioral observations were compatible with those inferred from polytene chromosome synapsis and allozyme studies by previous researchers. Moreover, Crossley (1986) reported that all species of the same complex have similar yet distinct courtship patterns and males of all species sing two songs: a long song early in courtship and a short song late in courtship. To elucidate the mechanisms of reproductive isolation, we reexamined the courtship behavior of intraspecific and interspecific mating and the yield of hybrids in the *D. bipectinata* complex.

Materials and Methods

An experimental base population of each local population was established by mixing eight or 10 isofemale lines. Flies collected in 1971 (Kitagawa, 1983) were maintained as isofemale lines in the laboratory. These species included *D. bipectinata bipectinata* (CB, Colombo, Sri Lanka), *D. parabipectinata* (BN, Bendar Seri Begawan, Brunei), *D. malerkotliana malerkotliana* (SP, Bukit Timah, Singapore), *D. malerkotliana pallens* (KC, Kuching, Malaysia), and *D. pseudoananassae nigrens* (KD, Kandy, Sri Lanka). A single-base population for each species or subspecies was used in the current study. After several generations, inseminated females were randomly selected from the base population and placed in vials (20 females per vial) and allowed to oviposit for 12 h. In this experiment, females and males that emerged from the vials were sexed and separated without etherization. The flies were reared and aged in a standard cornmeal–molasses medium. All experiments were performed at 25°C and under continuous light. The age of sexual maturity and the courtship behavioral pattern of each species, as well as the degree of reproductive isolation between populations, were measured and observed as follows.

Direct Observation to Measure Sexual Maturity

Twenty female and 20 male flies of the same age sexually separated within 15 h of eclosion were placed in an empty standard glass vial with a silted foam rubber plug. At the same time, five vials were set up, each containing five species and subspecies. The number of mated pairs was recorded for 30 min, and each mated pair was removed with an aspirator. Next, five replicates were produced for 4–9 day-old flies, two for 10-day-old flies, and one for 2–3 day-old flies.

Direct Observation of Courtship Behavior

Pair mating for courtship components

Virgin flies were collected from the base populations used to study sexual maturation. Next, 6–10-day-old virgin females and males from the same population were both placed in a small plastic cell (top diameter 30 mm, bottom diameter 20 mm, and height 4.5 mm). Several pairs were set up simultaneously, and the early courting pair was continuously observed from the onset of courtship until copulation. The duration and sequence of each aspect of male courtship, wing display, circling, and attempted copulation within the display were recorded simultaneously using an event recorder. The Student–Newman–Keuls test (Steel and Torrie, 1960) was used for statistical comparisons.

Mass Mating: No Choice for Courtship Classification

Virgin flies, 6–10 days old, 30 females, and 20 males, either from the same or from a different base population, were placed in an observation chamber (94 mm square, 17 mm high; Petit *et al.* 1976). Courtship was directly observed during a period of 30 min, and courtship bouts were recorded according to their classification as courtships without an attempted copulation; and courtships with successful copulation. Five replicates were made for each combination, and 100 males were observed.

Results and Discussion

Rate of Sexual Maturation

The rate of sexual maturation, as determined by insemination or mating, appeared to differ between species. Through direct microscopic observation of the spermathecae and seminal vesicles of dissected females, spermatozoa were first found at 28 h in *D. bipectinate bipectinata*, *D. parabipectinata*, and *D. malerkotliana malerkotliana*, but at 36 h in *D. malerkotliana pallens* and *D. pseudoananassae nigrens*. At 44 h, 81.5% of *D. bipectinate bipectinata* females were inseminated and appeared to reach full sexual maturity faster than the other species. In contrast, *D. pseudoananassae nigrens*, with 17.6% inseminated at 44 h, was the slowest maturing of these four species. More than 94% of the females of all species were inseminated after 92 h, and no significant differences were found between the species. Therefore, all members of the *D. bipectinata* species complex were considered mature at 4 days of age, and we used sexually isolated flies 5–10 days old in the following experiments.

Basic Courtship Pattern of Each Species and Subspecies

Basic courtship patterns are summarized in Table 1 using terminology according to Spieth (1952, 1966), Spieth and Ringo (1983), and Greenspan and Ferveur (2000). The outline of the courtship pattern of the *D. bipectinata complex* was as follows: the male moved behind the female, placed its head under the female's wings, and pushed her wings up with its head. The male displayed several types of wing movements, such as wing vibration and scissoring. All males, except for *D. bipectinata bipectinata* circled the female when she was unreceptive, especially in *D. pseudoananassae nigrens*, in unsuccessful pairs. While circling, the male performed special postures in front of the female, such as flapping one or both wings, bobbing its body up and down, arching its body back and forth, and moving in a crab-like sideways motion around the female's body. This circling behavior appeared to be the same as that frequently observed in *D. ananassae* (Spieth, 1966). During courtship that led to attempted copulation, males of *D. parabipectinata* and *D. pseudoananassae nigrens* usually positioned themselves in front of the female and circled her with a crab-like movement until they reached the rear of the female. The male of *D. malerkotliana* often circled the female in

a 360º arc to prevent the female from escaping and occasionally performed posturing actions in front of the female.

The copulatory actions observed for the *bipectinata* complex are classified into three types (Table 1). In *D. bipectinata*, males mounted the female after attempting to copulate. Another distinct type is characterized by the *D. malerkotliana* male where the attempt to mount follows achieved genital contact with the female, whereas a mixed type was found in D*. parabipectinata* and *D. pseudoananassae nigrens*. After achieving copulation, the male usually closes its wings, and the copulated pair remains stationary and then moves back and forth. In *D. bipectinata*, the male was in a forward position and the female was in a backward position; termination of copulation began with withdrawal and ended with dismounting, which was clearly characteristic of *D. bipectinata*.

Table 1. Mating Behavior Elements Recorded in the *D. bipectinata* Species Complex

Male action	*D. bipectinata bipectinata*	*D. parabipectinata*	*D. malerkotliana malerkotliana*	*D. malerkotliana pallens*	*D. pseudoananassae Nigrens*
Position of male posturing	rear	rear	rear	rear	Rear
Posturing movement					
a)Wing vibration	+	+	++	++	+
b) Scissoring	++	++	+	+	++
Circling	−	+	+++	++	±
a) Completely circles female	−	±	+	+	±
b) Posturing display	−	+	+	+	+
Wing movement with each element	L(sc)	L(sc)	L(wv)	L(wv)	L(sc)
	(wv)	(wv)	(sc)	(sc)	(wv)
	AC(sc)	AC(sc)	AC(wv)	AC(wv)	AC(sc)
Copulatory actions	m → cop	m → cop	cop → m	cop → m	m → cop
		cop → m			cop → m
Position of male	forward	rearward	rearward	rearward	Rearward
Dismounting	wd → dm	dm → wd	dm → wd	dm → wd	dm → wd

L: Licking, sc: scissoring, wv: wing vibration, AC: Attempted copulation, m: mounting, cop: copulation, wd: withdrawing, dm: dismounting. Terminology after Spieth (1952, 1966)

The court records for pairs that were successfully copulated are summarized in Table 2. The mean duration of courtship varied among species from 36.7–462.3 s. During this time, males performed an average of 1.2–4.6 courtship bouts. *D. malerkotliana malerkotliana* spent the longest time, significantly longer than *D. bipectinata* and *D. malerkotliana pallens* ($P < 0.05$): an average of 462.3 s until copulation, which consisted of 4.6 courtship bouts, each lasting an average of 64.6 s. Both the mean duration of the display and copulation attempts were similar between species, except for *D. bipectinata* males ($P < 0.05$), which successfully copulated with a single bout of courtship and no circling. However, only *D. malerkotliana malerkotliana* showed significant differences in the mean frequencies of any of the other four courtship elements ($P < 0.05$) for no courtship bouts, the number of circles, and the duration of copulation.

As shown in Table 2, successful *D. malerkotliana malerkotliana* courtship consisted of an average of 4.6 courtship bouts and 10.6 circles, whereas there was an average of 1.4 courtship bouts and 0.8 average circles for *D. malerkotliana pallens*. Furthermore, in *D. bipectinata* and *D. malerkotliana pallens* the mean duration of copulation was longer than that of the other three species and subspecies ($P < 0.05$). Additionally, no qualitative differences were observed between the two *D. malerkotliana* subspecies.

Consistent with Hegde and Krishnamurthy (1979) and Crossley (1986), the basic courtship patterns observed included circling differentiation in *D. malerkotliana* and low mating success in *D. pseudoananassae*

nigrens. However, although they reported that *D. parabipectinata* were similar in mating behavior, in the present study *D. bipectinata* displayed characteristic copulatory behavior, whereas the courtship behavior of *D. parabipectinata* seemed similar to that of *D. pseudoananassae nigrens* (Tables 1 and 2).

Table 2. Mean unit time and frequency of the courtship element.

species/ elements	N	A	B	C	D	E	N	F	G
D. bipectinata bipectinata	10 mean frequency	36.7 ± 21.9	16.3 ± 3.5 1.2	14.0 ± 3.7 1.1	0	13.1 ± 4.4 1.1	11	806.0 ± 51.2	400~1,040
D. parabipectinata	10 mean frequency	149.8 ± 43.4	30.0 ± 5.6 2.8	5.5 ± 0.6 7.6	5.0 ± 0.0 0.3	2.3 ± 0.4 2.9	18	532.0 ± 29.7	320~741
D. malerkotliana malerkotliana	10 mean frequency	462.3 ± 166.8	64.6 ± 13.7 4.6	4.4 ± 0.4 27.8	5.6 ± 0.8 10.8	1.3 ± 0.1 12	16	529.8 ± 22.1	400~695
D. malerkotliana pallens	10 mean frequency	51.7 ± 19.6	30.0 ± 10.7 1.4	6.4 ± 2.0 3.2	5.4 ± 0.8 0.9	2.4 ± 0.3 2.1	20	826.0 ± 40.7	555~1,245
D. pseudoananassae nigrens	10 mean frequency	263.1 ± 157.5	36.8 ± 9.6 2.4	5.1 ± 0.7 7.1	0	2.4 ± 0.7 2.1	21	515.7 ± 15.5	400~650

N: Number of pairs observed. Figures are mean unit length ± SE, in seconds mean frequency: mean frequency of each element
A: Mean time from the beginning of courtship to copulation
B: Court time C: Wing movement D: Circling E: Attempted copulation
F: Duration of copulation G: Range of duration of copulation in seconds

Courtship Behavior in InterSpecific Mating

Upon observation, the male was usually observed performing the species-specific courtship display to the female of any origin. Table 3 shows the number of occurrences in 100% during the 30-min observation period in both intraspecies and interspecies combinations. A high rate of courtship, including successful copulation, has been observed between the two subspecies of *D. malerkotliana*, comparable to the interspecies test. However, *D. malerkotliana pallens* males courted and attempted copulation less frequently than *D. malerkotliana malerkotliana* males. Although no copulation was observed in the opposite cross, successful interspecific copulation was found in the combinations of *D. malerkotliana malerkotliana* females with *D. bipectinata* males and *D. bipectinata* females with *D. parabipectinata* males. Notably, the males of *D. bipectinata* never showed courtship with copulation attempts in the latter cross combination.

The relationship between the total number of courtship bouts in mass mating and the percentage of mated pairs in pair mating are presented in Tables 3 and 2, respectively. In intrapopulation combinations that resulted in successful copulation, the average number of total courtship bouts ranged from 43 in *D. pseudoananassae nigrens* to 152 in *D. parabipectinata*. In *D. malerkotliana*, there were four intraspecific cross combinations. *D. malerkotliana pallens* courted and inseminated more *D. malerkotliana malerkotliana* females than females of their own species. Furthermore, this result showed a lower frequency of copulation under the mass mating condition (Table 3). However, *D. malerkotliana malerkotliana* males were accepted by *D. malerkotliana pallens* as often as their own subspecies. In contrast, interspecific attempts that were unsuccessful in pair mating exhibited less than 100 total bouts in mass mating. The mean duration between intraspecific combinations (Table 3) was significantly different ($P < 0.05$). These interspecific or intersubspecific combinations were significantly different from both parental intraspecific combinations at the 5% level using Dunnett's procedure (Steel and Torrie, 1960).

Interspecific cross combinations showed strong, asymmetric sexual isolation between species. *D. pseudoananassae nigrens* showed complete reproductive isolation from other species (Aotsuka and Tobari, 1983; Kopp and Barmina, 2005), but females of *D. pseudoananassae nigrens* accepted males from *D. bipectinata* and *D. parabipectinata*. In crosses with males of *D. bipectinata*, which exhibit characteristic copulatory behavior, the isolation between all other species except *D. pseudoananassae nigrens* was stronger than that between reciprocal males. Therefore, a lower number of courtship displays are associated with strong sexual isolation.

Table 3. Several bouts and percent of insemination among intercrossed between the *bipectinata* complexes

Male	*D. bipectinata bipectinata,* CB		*D. parabipectinata*, BN		*D. malerkotliana malerkotliana*, SP		*D. malerkotliana pallens*, KC		*D. pseudoananassae nigrens*, KD	
Female	No. of bouts	% of insemination	No. of bouts	% of insemination	No. of bouts	% of insemination	No. of bouts	% of insemination	No. of bouts	% of insemination
D.bipectinata bipectinata	77	100	174	75.7	224	2.7	183	2.2	133	0
D.parabipectinata	136	57	152	100	82	0	49	0	62	0
D.malerkotliana malerkotliana	207	2.8	122	2.9	140	100	168	100	37	0
D.malerkotliana pallens	142	11.4	103	12.5	192	100	121	94.1	74	0
D.pseudoananassae nigrens	106	2.7	144	11.1	136	0	58	0	43	100

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References: Aotsuka, T., and Y.N. Tobari 1983, Report on the overseas scientific expedition for the collection of Drosophilid flies 1971-1982. (ed. O. Kitagawa). 62-72; Bock, I.R., 1971, Univ. Texas Publ. 7103: 273-280; Bock, I.R., 1980, Syst. Entomol. 5: 341-356; Crossley, S.A., 1986, Anim. Behav. 34: 1146-1159; Greenspan, R.J., and J.F. Ferveur 2000, Annu. Rev. Genet. 34: 205-232; Hegde, S.N., and N.B. Krishnamurthy 1979, Aust. J. Zool. 27: 421-431; Kitagawa, O., 1983, The overseas scientific expedition for the collection of Drosophilid flies 1971-1982. (ed. O. Kitagawa). Tokyo, Japan; Kopp, A., and O. Barmina 2005, Genet. Res. 85: 23-46; Matsuda, M., Y. Tomimura, and Y.N. Tobari 2005, Genetica 125: 69-78; Petit, C., O. Kitagawa, and T. Takamura 1976, Jpn. J. Genet. 51: 99-108; Singh, S., and B.N. Singh 2001, Indian J. Exp. Biol. 39: 835-844; Spieth, H.T., 1954, Bull. Am. Mus. Natur. Hist. 99: 395-474; Spieth, H.T., 1966, Univ. Texas Publ. 6615: 133-145; Spieth, H.T., and J.M. Ringo 1983, In: Ashburner, M., H.L. Carson, and J.N. Thompson, jr. (eds.) *The Genetics and Biology of* Drosophila 3c. Academic Press, London. 223-283; Tomimura, Y., M. Matsuda, and Y.N. Tobari 2005, Genome 48(3): 487-502; Val, F.C., and F.M. Sene 1980, Papeis Avulsos Zool. 33: 293-298.

Cacti necroses as overwintering microhabitats of drosophilids in Valle Fértil Natural Reserve (San Juan, Argentina).

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The Natural Reserve of Valle Fértil, located in San Juan province in Northwestern Argentina, is a humid valley surrounded by much drier areas. The exceptional humidity conditions make this place the most biodiverse of the region. Within the Reserve, a particularly rich microhabitat for arthropods are the decaying tissues of the cacti (Padró *et al.,* 2023). Despite more than a decade of ongoing monitoring, no winter fieldwork had been conducted in the area until recently. In early September 2023, a winter field trip was undertaken with the objective of characterizing the Drosophilidae community during this seasonal period. It is well-established in the literature that various *Drosophila* species exhibit differential cold resistance (Cobb *et al.,* 2021; Stazione *et al.,* 2020; Trotta *et al.,* 2006; Hoffman *et al.,* 2003; David *et al.,* 1998). However, the majority of these studies have been conducted under laboratory experimental conditions (Mensch *et al.,* 2017; Anderson *et al.,* 2005; Krebs and Loeschcke, 1994), and only a limited number have been carried out in natural environments. Furthermore, a small proportion of these studies have been conducted in southern South America (Alruiz *et al.,* 2022; Sørensen *et al.,* 2005). A prevalent assumption might be that adults would be absent and larvae would be in a state of arrested development within the decaying tissue.

Sampling was conducted using traps baited with fermented banana, following the protocol outlined by Markow and O'Grady (2006). These traps were positioned adjacent to *Trichocereus terscheckii* individuals for a duration of two days. Flies were collected from the traps during both morning and evening sessions and subsequently transferred to tubes containing DESS preserving solution, as detailed by Pavlovska *et al*. (2021). Concurrently, we conducted inspections for active necroses and examined the tissue for the presence of larvae and adults. The latter were captured using nets. Taxonomic identification was later performed in the laboratory.

Table 1. Number and percentage of individuals in each group of the sample.

Taxa	Number	Percentage	Species
buzzatii cluster (cactophilic Drosophila)	15	71.43%	*D. koepferae* *D. buzzatii*
non cactophilic Drosophila	5	23.81%	*D. nebulosa*
Neriidae family	1	4.76%	*Eoneria maldonadoi*
Total	21	100%	

Simultaneously, we measured both environmental and microenvironmental temperature by deploying data loggers inside the decaying pockets and the surrounding area (2 meters away from the focal specimen). These loggers were left in place for 48 hours.

Results and Discussion.

A total of twenty-one adult flies were collected and categorized into drosophilids from the buzzatii cluster (cactophilic), other non cactophilic drosophilids, and flies from the Neriidae family, as detailed in Table 1. Actively flying adults, both nerids and drosophilids, were also observed around rotting pockets in the warmer periods of the day.

Fifteen necrotic pockets were identified in the area comprising both columnar cacti (*T. terscheckii*) and cladodes of prickly pears (*Opuntia sulphurea*). The majority of these rots exhibited drosophilid larvae in various stages of development, along with pupae.

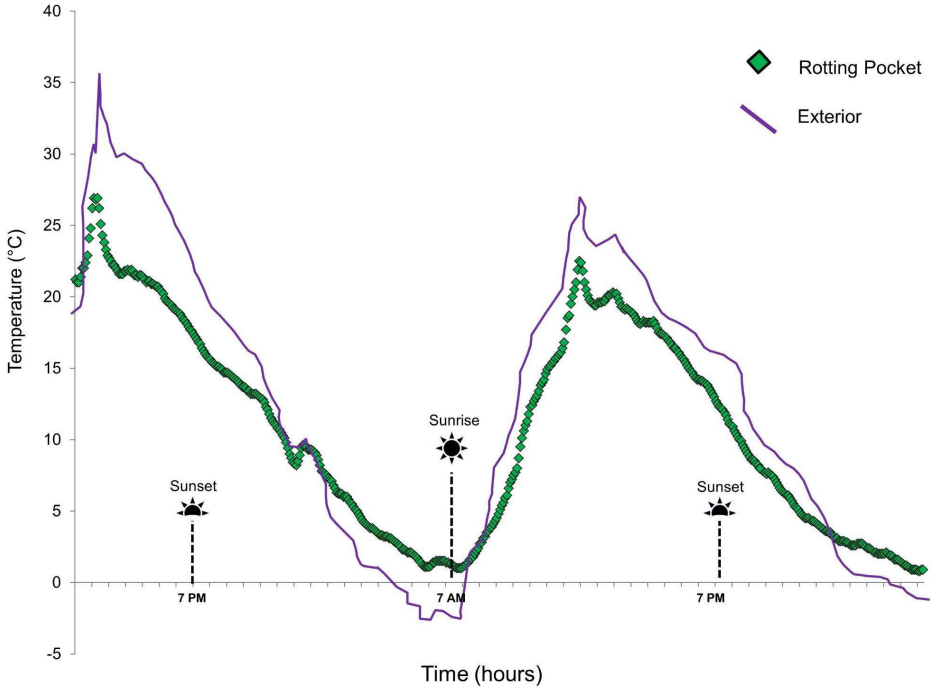

Figure 1. Temperature fluctuations during the day recorded with data loggers placed inside a rotting pocket of *Trichocereus terscheckii* (green points) and in the vicinity (2 meters away) of the specimen (solid purple line).

Regarding temperature measurements (Figure 1), a significant thermal amplitude was observed in the environment, with daytime maximum reaching 36°C and nighttime minima dropping to minus two degrees Celsius in both recorded nights. However, within the necrotic pocket, the temperature exhibited a buffered variation, not exceeding 27°C at its maximum and never reaching the freezing point during the coldest moments of the night. Our data would suggest that, in addition to providing sustenance, columnar necroses may serve as thermal refuges in winter, enabling the presence of adults in populations even during the coldest months of the year. Subsequent efforts will focus on expanding sampling points and incorporating *Opuntia* necroses into monitoring activities to ascertain if differences exist among various natural hosts.

References: Alruiz, J.M., I. Peralta-Maraver, F. Bozinovic, M. Santos, and E.L. Rezende 2022, J. Anim. Ecol. 91: 655–667; Anderson, A.R., A.A. Hoffmann, and S.W. McKechnie 2005, Genet. 85(1): 15–22; Cobb, T., D. Damschroder, and R. Wessells 2021, Insect Biochem. Mol. Biol. 133: 103548; David, R.J., P. Gibert, E. Pla, G. Petavy, D. Karan, and B. Moreteau 1998, J. Therm. Biol. 23(5): 291-299; Hoffmann, A.A., J.G. Sørensen, and V. Loeschcke 2003, J. Therm. Biol. 28(3): 175-216; Krebs, R.A., and V. Loeschcke 1994, EXS 68: 309–321; Markow, T.A., and P. O'Grady 2006, A.P. 1-272; Mensch, J., J. Hurtado, P.F. Zermoglio, G. de la Vega, C. Rolandi, P.E. Schilman, T.A. Markow, and E. Hasson 2017, J. Exp. Biol. 220(4): 713–72; Padró, J., A. Saint Esteven, and I.M. Soto 2023, Biodivers. Conserv. 32(2): 567-587; Pavlovska, M., I. Prekrasna, I. Parnikoza, and E. Dykyi 2021, Microbes Environ. 36(1); Sørensen, J.G., F.M. Norry, A.C. Scannapieco, and V. Loeschcke 2005, J. Evol. Biol. 18(4): 829–837; Stazione, L., F.M. Norry, F.H. Gomez, and P. Sambucetti 2020, Ecol. Evol. 10(4): 1998–2006; Trotta, V., F.C. Calboli, M. Ziosi, D. Guerra, M.C. Pezzoli, J.R. David, and S. Cavicchi 2006, BMC Evol. Biol. 6: 67; Yilmaz, M., and E.D. Özsoy 2022, EJE 119: 140-147.

Cold-induced aneuploidy of the 4th-chromosome in *Drosophila melanogaster*.

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Mitosis and meiosis are usually taught in introductory biology courses, but students often find these topics boring when they are forced to memorize the stages thereof. Teachers who rely on rote learning may not realize that these processes are tiny marvels of mechanical engineering, equipped with exquisite gadgetry (Gerhart and Kirschner, 1997). In particular, the spindle apparatus is the cellular equivalent of a death-defying, high-wire circus act: one slight misstep can kill you! If conjoined chromosomes fail to separate properly after metaphase—an error known as "nondisjunction"—the daughter cells receive extra or missing chromosomes (Held, 1982). Such imbalances are termed "aneuploidy" (Orr *et al*., 2015), and when they occur during meiosis in women, they typically cause lethal miscarriage of the embryo (Strauss *et al.*, 2023).

The human 21st chromosome is an exception to this rule. It is so small that it can tolerate trisomic dosage without killing the fetus, though the resulting "Down Syndrome" entails severe mental impairment (Hassold and Hunt, 2001). The fruit fly's 4th chromosome is likewise tiny (Riddle and Elgin, 2018), and it can similarly withstand aneuploidy without killing the embryo (Wakimoto *et al*., 2004). To wit, triplo-4 adults are nearly normal (Ashburner, 1989), while haplo-4 flies manifest a syndrome of thin bristles, abnormal wing venation, and female sterility (Lindsley and Zimm, 1992). Given these parallels between flies and humans, it should be easy to devise laboratory exercises using the fruit fly to illustrate the etiology of Down Syndrome. Here we offer such an exercise that is both simple and cheap. Moreover, the data that it generates present opportunities for insightful discussions of cellular mechanisms.

Our experimental design is based on the ability of temperatures near 0˚C to dissolve microtubules (Li and Moore, 2020) and thereby disable spindle function (Steven *et al.*, 2016). Comparable investigations were performed by Bauer (1946), who exposed fly embryos to freezing temperatures, allowed them to develop into adults, and examined their offspring for evidence of polyploidy. Subsequently, Woodruff and Thompson (2002) exposed female flies to 4-5˚C and screened their offspring for evidence of aneuploidy affecting the X chromosome. We used the same cold treatment as Bauer, but we scrutinized the exposed individuals *themselves* (not their progeny) for evidence of aneuploidy in their imaginal epidermis. Thus, our method differs from both earlier approaches insofar as it spans only two fly generations, instead of three.

Materials and Methods

The fly strain that we used carries the recessive mutation *shaven-depilate* (sv^{de}) on one of its 4th chromosomes and *eyeless-Dominant* (ey^{D}) on the other (Lindsley and Zimm, 1992): sv^{de}/Dp(2:4) ey^{D} (Kronhamn *et al.*, 2002). It was supplied by the Bloomington Stock Center (#662) and cultured on Ward's *Drosophila* Instant Medium with baker's yeast grains sprinkled on top. This stock is stable in heterozygous condition because ey^{D}/ey^{D} homozygotes die as larvae, and sv^{de}/sv^{de} homozygotes are sterile. The sv^{de}/ey^{D} heterozygotes, which predominate, have small eyes and enlarged sex combs, while the less frequent sv^{de}/sv^{de} homozygotes lack bristles over most of their body—whence the name "shaven"—though bristle sockets typically persist at sites where shafts are missing (Held and Harrington, 2022). Novices unfamiliar with fly genetics can consult Roote and Prokop's (2013) training manual to acquaint themselves with the basics.

Parental sv^{de}/ey^{D} flies were placed in plexiglass egg-laying cages (Genesee Scientific) fitted with a 0.135-mm mesh at one end and an agar-filled petri dish (3.5-inch diameter) at the other, which was held snugly in place with a flexible plastic collar (Figure 1a). Petri dishes were poured with a solution of Bacto™ Brain Heart Infusion (BHI) powder (37 g/liter) and Bacto™ Agar (15 g/liter; both ingredients from Benton, Dickinson, & Co., Sparks, MD) autoclaved at 121˚C for 30 mins. After cooling to room temperature, the plates were sprayed with Tegosept (Ward's Science) anti-fungal solution (25 g/250 ml ethanol) and painted with active yeast paste (Fleischmann's) on half their surface to stimulate egg-laying. We fitted each cage with a

plate and placed them in a 25˚C incubator, tilted at a slight angle (Figure 1b) so that any falling adults would not land on the paste.

Eggs were collected for 2-h periods, whereupon we replaced the old plates with fresh ones. Egg-containing plates were then floated on a 0˚C ice bath (experimental) or a 25˚C bath (control) for 2 h (Figure 1c), after which eggs were counted (Figure 1d), and plates were returned to the 25˚C incubator to allow larvae to hatch. Those larvae were transferred to food bottles for the duration of development, after which the eclosed adults (preserved in 70% ethanol) were inspected for sv^{de}/- mosaic patches (at 50X magnification) resulting from loss of the ey^{D} homolog, using a Nikon dissecting microscope. Heterozygotes were occasionally found with isolated cases of empty sockets, indicative of a sv^{de}/- genotype, in both the control and experimental series. However, such flies were not scored as early-loss mosaics for our analysis, since tiny spots likely arise by the kind of late-loss nondisjunction that affects the X chromosome in gynandromorphs (Held, 1979; Ripoll, 1972).

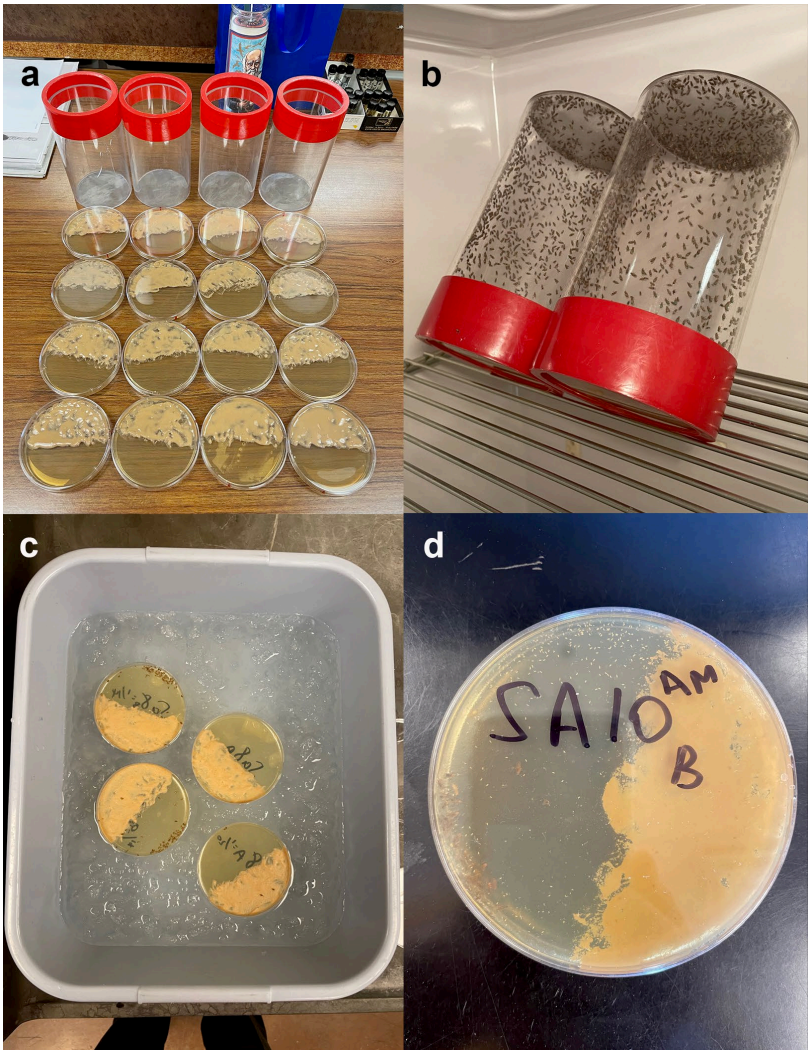

Figure 1. Egg collection and cold treatment. **a.** Plexiglass egg-laying cages (top) and agar plates with yeast paste on half of their surface (bottom). **b.** Two such cages full of flies (mesh at top) were tilted so that any flies which slip down the sides would fall onto clean agar, rather than onto sticky yeast paste. **c.** Four petri dishes floating (yeast side up) on an ice bath. Note the pile of dead flies at the edge of the two plates on the left, where they had slipped and fallen onto the area devoid of yeast. **d.** Agar plate (yeast side down) placed on a black table to allow counting of the eggs (white dots). The collection period (Saturday starting at 10AM, cage B) is marked.

Results and Discussion

We used four egg-collection cages, each filled with approximately 2,000 sv^{de}/ey^{D} adults, to lay eggs for five 2-h periods (one set of four agar plates per period) each day for two days. Only ~10% of the eggs hatched from the cold-treated plates, but the control percentage was not much higher (~15%; sample N = 1,345 eggs).

Among 2,207 experimental adults, we found 1,821 sv^{de}/ey^{D} heterozygotes (863♂; 958♀), 380 sv^{de}/sv^{de} homozygotes (176♂; 204♀), and 6 mosaics (3♂; 3♀). Hence, the overall frequency of mosaics was 6/2207 or 0.27%, which agrees remarkably well with the 0.26% (18/7000) aneuploids recovered by Woodruff and Thompson (2002) and the 0.265% (21/7924) triploid offspring found by Bauer (1946) after he exposed 2-h-old eggs to 2-h pulses of 0˚C. To calculate the frequency of nondisjunction per se (6/1821 = 0.33%), we must exclude sv^{de}/sv^{de} siblings, whose sv^{de}/- patches would blend into the cuticular background. Five of our six

mosaics are shown in Figure 2. Among the controls (not exposed to cold), we found a single mosaic among 2105 flies. It had a patch of ~12 empty sockets in the dorso-central area of its left hemi-notum.

The sv^{de}/- patches obtained by chilling embryos offer a panoply of puzzles for students to solve, either by (1) speculating on their own, (2) collaborating with lab mates, or (3) conversing with the instructor. In order for students to construct plausible etiologies for patch peculiarities, they must acquire a thorough grasp of fly development (Hartenstein, 1993), which can only be forged through further study. Ideally, exposing students to such riddles will launch them into the literature of fly genetics with the passion of a pirate who is keen on seeking buried treasure.

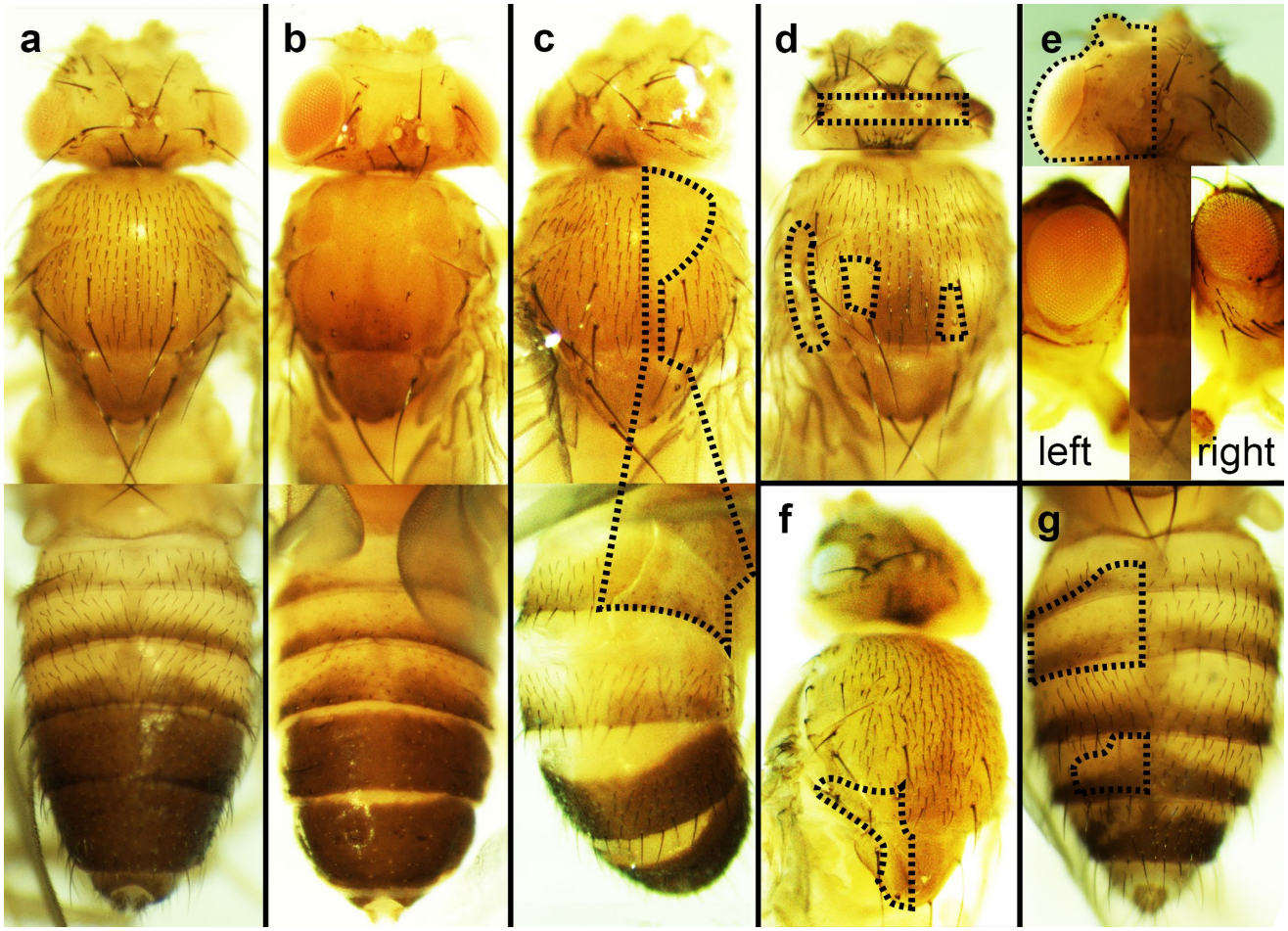

Figure 2. Normal (**a**, **b**) and genetically mosaic (**c**-**g**) adults that grew from cold-treated eggs. (Upper and lower panels in **a**-**c** are the same fly at different focal planes, and the same is true for head versus thorax in the upper and lower parts of panel **d**.) **a.** Heterozygous sv^{de}/ey^{D} male. Note the small eyes due to the ey^{D} mutation. **b.** Homozygous sv^{de}/sv^{de} male. Virtually all bristle shafts on the thorax and abdomen are missing except for two scutellar macrochaetes (the phenotype is variable), while most of the sockets remain (visible as brown dots that are more prominent on the tergites). In contrast, the head retains an assortment of bristles that varies from fly to fly. **c**-**g.** Mosaic flies, whose bodies are mostly sv^{de}/ey^{D}, but which display discrete patches of sv^{de}/- tissue at various locations, circumscribed by dotted lines around their perimeters. **c.** Mosaic male with the largest patch of sv^{de}/- tissue that we found. The stripe of bald cuticle on the fly's right side stretches from the front of its notum to the rear of its 3rd abdominal tergite, abutting the body midline but varying in width. Note sockets in bare areas, more obvious on the scutellum and tergites, less so on anterior notum. **d.** Mosaic male with a symmetric patch of sv^{de}/- tissue straddling the head's midline and three isolated bald spots on the notum. **e.** Mosaic female, the left side of whose head is missing virtually all shafts. Insets show left and right sides of the head. The left eye is normal in size (consistent with sv^{de}/-) and is smooth due to an absence of inter-

ommatidial bristle shafts, while the right eye has an *ey*^{D} (small) phenotype. Note also shaftless sockets along the ventral rim of the left eye. **f.** Mosaic female with a patch of *sv*^{de}/- tissue in the left rear of its thorax with reduced or missing shafts. The anterior scutellar macrochaete is present, but it could be *sv*^{de}/- because such bristles show variable expressivity in mutant flies (**b**). **g.** Mosaic female with *sv*^{de}/- tissue on the left side of its 2nd (partial), 3rd (total), and 5th (partial) tergites, all of which abut the midline.

For example, the male in Figure 2c has a contiguous patch of *sv*^{de}/- tissue on its right side that extends from the front of its thorax to the rear of its 3rd abdominal tergite. What does this mean? To answer such questions, students must realize that a fly's adult surface is formed from islands of cells—"imaginal discs" or "abdominal histoblasts"—embedded in the embryonic ectoderm (Held, 2002). The "fate map" of those structures offers clues that are essential for solving the mystery. To wit, imaginal discs on the left and right sides of the body start out widely separated and only fuse at the midline during metamorphosis, when the intervening (larval) cells are eliminated by cell death. Hence, patch boundaries should often be sharp at the midline, but ragged elsewhere (Hotta and Benzer, 1972), as is evident not only in Figure 2c, but also in Figs. 2e and 2g. (The patch in Figure 2f only hugs the midline in the scutellum, not in the notum proper.)

The male in Figure 2d has a strip of *sv*^{de}/- tissue straddling the midline, which crosses from one eye disc to the other, while its other three patches are about the same size, but located on the left and right sides of the notum. How does patch size relate to the timing of nondisjunction? To figure this out, students must grasp the inverse correlation between these variables. Namely, the earlier that spindles dissolve during the 2-h window of cold treatment, the larger the patches should be, and conversely, the later that they dissolve, the smaller they should be. Moreover, students should understand that fly embryos undergo an odd type of cleavage where karyokinesis is uncoupled from cytokinesis, and mitoses are synchronized for 13 cycles (Foe, 1989). Because a cold shock could disable spindles at disparate sites simultaneously, contiguity in adult skin does not necessarily indicate descent from a common ancestral spindle (Hartenstein *et al.*, 1995). That is what may have happened in this case, and the same logic applies to the mosaic in 2g.

Alternatively, the two patches on the left side of Figure 2d could have come from the *same* nuclear ancestor but later split apart due to the phenomenon of cell competition (Simpson, 1981). Haplo-4 cells only have one copy of a *Minute* gene located on the 4th chromosome (Lindsley and Zimm, 1992), so *sv*^{de}/- cells grow more slowly than neighboring *sv*^{de}/*ey*^{D} cells in the same disc. The ensuing rivalry could fragment a putative *sv*^{de}/- clone into detached spots by extrusion of intervening *sv*^{de}/- cells (Nagata and Igaki, 2018). This scenario cannot explain the two patches in Figure 2g, however, because cells in separate histoblast nests would not compete. Thus, the patches anterior and posterior to the 4th tergite must have arisen by separate nondisjunction events.

The female in Figure 2e displays a stark asymmetry in its head. The left side has a normal (non-*ey*^{D}) eye and is virtually devoid of bristle shafts, while the right side has a reduced (*ey*^{D}) eye and retains intact bristles. Evidently, the left eye disc arose from contiguous *sv*^{de}/- (aneuploid) nuclei in the embryonic ectoderm, while the right eye disc resided in normal (euploid) territory. Another oddity is that the sole head bristle on the left is thinner than its counterpart on the right. This atrophy could be due to *sv*^{de} alone, but it could also arise from 4th chromosome loss (*sv*^{de}/-) that removes a ribosomal gene, leading to a haplo-insufficient "Minute" phenotype (Marygold *et al.*, 2007). The same effect might also explain the thin thoracic bristles of the mosaic in Figure 2f.

We did not screen for normal eyes among otherwise *sv*^{de}/*ey*^{D} heterozygotes, so the mosaic in Figure 2e would have escaped our notice if it had not been missing bristles on the top of its head. Other kinds of aneuploid patches would also have eluded our "radar." Based on the 0.27% frequency of haplo-4 patches, roughly 3 (0.33%) of our 958 cold-treated *sv*^{de}/*ey*^{D} females should have become gynandromorphs via loss of an X chromosome as embryos. We chose not to look for such mosaics because that would have required us to meticulously scrutinize putative females for telltale (1) sex combs on one or both forelegs or (2) black spots on rear tergites. Moreover, based on Bauer's (1946) tallies of triploid offspring, we would have expected to find patches of tetraploid (4n) cells on roughly 6 (0.33%) of our 1821 *sv*^{de}/*ey*^{D} flies. The only place on the body where such spots would be detectable at the magnification of an ordinary dissecting microscope like the one we used for our survey would be the wings, where 4n patches would show up as light areas due to low density

of wing hairs (Held, 2003). In order to assess whether such events occurred, we inspected both wings of 230 cold-treated sv^{de}/ey^{D} flies and found a single male which met the above criteria. We would not expect neophytes to conduct such tedious screens successfully, so we did not pursue this line of investigation further.

Aside from the side-benefit of prodding students to study fly development in order to interpret their results, this exercise could spark curiosity about spindle vulnerability, which poses a counterintuitive paradox: why should microtubules dissolve when cooled (Li and Moore, 2020) given the *greater* stability of most protein structures at lower temperatures? Asking students to grapple with this enigma could lead them to inquire more deeply into self-assembly mechanisms.

Fittingly, this year marks 100 years since Harvard entomologist William Morton Wheeler penned a manifesto entitled "The dry rot of our academic biology" (Wheeler, 1923). In that essay he railed against the kinds of "cook book" laboratory exercises widely used in biology classes. Instead, he advocated hands-on engagement with living organisms as a way of imbuing students with an appreciation for the natural world. Our protocol is inherently consistent with that goal.

Letting students deduce the etiologies of fly anomalies effectively extracts the instructor and turns a teaching lab into a research lab where students become independent investigators. Such exploratory "microworlds" were championed by Seymour Papert (1980), LIH's mentor, and adapted for cell biology classes by SJB (Banerjee *et al.*, 2021) in accord with the CURE (Course-based Undergraduate Research Experiences) methodology (Dolan and Weaver, 2021). Our coauthor apprentices (HAN and OFV) "field-tested" this approach, where learning occurs from the bottom up, vs. top down. Another example of this pedagogic strategy is offered in a companion paper (Held *et al*., 2023).

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References: Ashburner, M., 1989, Drosophila: *A Laboratory Handbook.* CSH Press, Cold Spring Harbor, New York; Banerjee, S.J., *et al.,* 2021, Sci. J. Biol. Life Sci. 1: 2020; Bauer, H., 1946, Zeitschrift für Naturforschung 1: 35-38; Dolan, E.L., and G.C. Weaver 2021, *A Guide to Course-based Undergraduate Research: Developing and Implementing CUREs in the Natural Sciences.* W.H. Freeman, New York; Foe, V.E., 1989, Development 107: 1-22; Gerhart, J., and M. Kirschner 1997, *Cells, Embryos, and Evolution.* Blackwell Science, Malden, Massachusetts; Hartenstein, V., 1993, *Atlas of* Drosophila *Development.* CSH Press, Cold Spring Harbor, New York; Hartenstein, V. *et al.* 1995, Trends Genet. 11: 51-58; Hassold, T., and P. Hunt 2001, Nature Rev. Genet. 2: 280-291; Held, L.I., Jr., 1979, W. Roux's Arch. 187: 129-150; Held, L.I., Jr., 1982, Mutat. Res. 94: 87-101; Held, L.I., Jr., 2002, *Imaginal Discs: The Genetic and Cellular Logic of Pattern Formation.*Cambridge Univ. Press, New York; Held, L.I., Jr., 2003, Dros. Inf. Serv. 86: 50-53; Held, L.I., Jr., and L.G. Harrington 2022, Dros. Inf. Serv. 105: 32-37; Held, L.I., Jr., *et al*., 2023, Dros. Inf. Serv. 106: 20-25; Kronhamn, J., *et al.,* 2002, Development 129: 1015-1026; Li, G., and J.K. Moore 2020, Molec. Biol. Cell 31: 1154-1166; Lindsley, D.L. and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Marygold, S.J., *et al.,* 2007, Genome Biol. 8: R216; Nagata, R. and T. Igaki 2018, Dev. Growth & Differentiation 60: 522-530; Orr, B., K.M. Godek, and D. Compton 2015, Curr. Biol. 25: R538-R542; Papert, S., 1980, *Mindstorms: Children, Computers, and Powerful Ideas.* Basic Books, New York; Riddle, N.C. ,and S.C.R. Elgin 2018, Genetics 210: 757-772; Ripoll, P., 1972, Roux's Arch. Dev. Biol. 169: 200-215; Roote, J., and A. Prokop 2013, G3: Genes, Genomes, Genetics 3: 353-358; Simpson, P., 1981, J. Embryol. Exp. Morph. 65 (Suppl.): 77-88; Steven, A.C., W. Baumeister, L.N. Johnson, and R.N. Perham 2016, *Molecular Biology of Assemblies and Machines.* Routledge, New York; Strauss, J.F., *et al.*, eds. 2023, *Yen & Jaffe's Reproductive Endocrinology*, 9th Ed. Elsevier, Amsterdam; Wakimoto, B.T., D.L. Lindsley, and C. Herrera 2004, Genetics 167: 207-216; Wheeler, W.M., 1923, Science 57: 61-71; Woodruff, R.C., and J.N. Thompson, Jr. 2002; Dros. Inf. Serv. 85: 149-151.

Freaky pharate flies from virgin mothers in *D. melanogaster.*

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Fruit flies look nothing like humans, but they are surprisingly similar to us genetically (Held, 2017). One of our shared features is our mode of sexual reproduction: in both cases, "XX" females mate with "XY" males to maintain a 50:50 ratio of males to females. Parthenogenesis is an alternative mode whereby eggs acquire the ability to proceed through development without ever being fertilized by a sperm (Simon *et al*., 2003). This phenomenon occurs rarely in humans in association with ovarian tumors (Hegazy *et al.*, 2023), but it happens more commonly in flies to produce viable adults (Sperling and Glover, 2023a). One well-studied parthenogenic species in the *Drosophila* genus is *D. mercatorum* (Thomalla and Wolfner, 2023), where a purely female population can perpetuate itself indefinitely.

Recently, Sperling *et al.* (2023) succeeded in (1) identifying three key genes responsible for virgin births in *D. mercatorum* and (2) using CRISPR to insert them into *D. melanogaster* so as to create a strain having the genotype *GFP-polo*$^{+}$; *Myc*^{dp} *Desat2*$^{-}$/*TM6B, Tubby*^{1}. We refer to this stock by the acronym "*pMD/TM6B.*" The *TM6B* balancer chromosome bears the dominant *Tubby*^{1} mutation (Craymer, 1984), which allows heterozygous *pMD/TM6B* pupae to be discerned from their *GFP-polo*$^{+}$; *Myc*^{dp} *Desat2*$^{-}$/ *Myc*^{dp} *Desat2*$^{-}$ homozygous siblings, based on their squat shape, which resembles a beer barrel (Lattao *et al*., 2011). *pMD/TM6B* females are more fertile than *pMD/MD* females (A. Sperling, personal comm.; confirmed by us), so we relied only on them for our egg collections.

How can an unfertilized egg develop without a sperm, given that haploidy is lethal for fly embryos (though haploid patches can survive in 1n/2n mosaic individuals; Santamaria, 1983)? In dipterans at least, the answer is that they recruit 1, 2, or 3 of the byproduct nuclei from meiosis ("polar bodies") to essentially function as "surrogate" sperm. These nuclei apparently fuse with the egg nucleus and thereby raise the ploidy above a haploid level (Sperling and Glover, 2023a). Consistent with this conjecture, Sperling *et al.* (2023) found a 6:5:1 ratio of 2n:3n:4n offspring in a karyotyped sample of 12 F_1 larvae that they tested from a group of *pMD/TM6B* virgin mothers.

We have long been interested in higher ploidy as a way of studying the effects of cell size on cuticular patterns (Held, 1979, 1982, 2003; *cf*. Fankhauser, 1945), so the chance to create 4n flies in this way was appealing (see Novitski, 1984). However, very few viable eggs are laid by virgin *pMD*/*TM6B* females. Starting with 21,406 virgins, Sperling *et al*. (2023) got only 143 F_1 offspring (~12 of which should have been 4n based on the 8.3% frequency in their sample), and those 143 F_1 virgins only yielded two F_2 adults! Sperling *et al*. did not save any polyploid adults (personal comm.), so we had to repeat their experiment at the same "brute-force" scale in order to harvest enough 4n flies for our analysis. Faced with the prospect of collecting 1000s of virgins, we (LIH and SJB) sought help from our students, who became our coauthors. Even with this team in place, we sought further ways to enhance the dismal yield of the *pMD/TM6B* strain, one of which was to employ "placebo" (spermless) males to spur greater virgin fecundity.

D. melanogaster females lay more eggs per day when mated than when unmated, but they can be coaxed to lay almost as many eggs by crossing them with spermless males that only transfer seminal fluid during copulation (Chapman *et al*., 2001; Heifetz *et al*., 2001). Spermless X/0 males that arise via nondisjunction (Ashburner, 1989; Orr *et al*., 2015) have virtually no effect on parthenogenesis in dipterans (Sperling and Glover, 2023). However, we suspected that other types of spermless males might have better success (Kalb *et al*., 1973; Wakimoto *et al*., 2004). Based upon prior work done by others, we decided to try using spermless males produced by the *tudor* mutation (Boswell and Mahowald, 1985; Xue and Noll, 2000), which blocks pole cell initiation in the embryo (Thomson and Lasko, 2004), leading to a grandchildless phenotype.

Materials and Methods

We ordered three different *tudor* strains from the Bloomington Stock Center (#1735, #1786, and #3243). No viable *tudor* females were recovered from #3243, and the ones that we obtained from #1735 were sterile. Only the #1786 stock proved useful. Its genotype is *tudor*^{1} *bw*^{1} *speck*^{1}/*CyO*. To create spermless males, we crossed brown-eyed, non-Curly, #1786 virgins with Oregon R males and tested the putatively spermless sons for actual sterility by mating them with virgin Oregon R females. After verifying that no larvae ensued after one week, we aspirated those males into fresh food vials containing virgin *pMD*/*TM6B* females to determine whether the introduction of such males could boost the frequency of viable F_1 offspring in subsequent weeks.

In all of our experiments, females were sorted from males at the pharate adult stage to ensure virginity with 100% confidence. (Sex combs, or lack thereof, can be clearly seen inside the pupal case after bristles are pigmented.) Only *Tubby* (*pMD*/*TM6B*) heterozygous females were utilized, since they are more viable and fertile than non-*Tubby* homozygotes (*pMD/MD*).

All flies were raised on blue *Drosophila* Instant Medium, Plain Formula 4-24 (Carolina Biological Supply), instead of the white version because the darker color allows eggs and pupae to be more easily detected by contrast with the background. Grains of active (live) dry yeast (Fleischmann's) were sprinkled on the food and wetted with drops from a squirt bottle before using a paintbrush to spread pupae on the inner walls of the vial. Egg-collection vials were kept in a 25˚C incubator (without any controlled humidity). The instant food lost moisture quickly, so we had to add water daily (with a Pasteur pipette) to prevent the mothers from dying by thirst.

Results and Discussion

We collected eggs from 67 cohorts of virgin *pMD*/*TM6B* females, with ~50 flies per vial (3,338 total), over a period of 1-2 months, transferring those females to fresh food once a week. Following removal of the flies from each vial, we examined the vial for the presence of F_1 pupae for two weeks. Every eclosed F_1 adult was mated with 8-10 wild-type (Oregon R) males to assess its ploidy (2n, 3n, 4n) based on F_2 phenotypes, while every pharate adult that failed to eclose was preserved in 70% ethanol and inspected for cuticular abnormalities. Figure 1 shows a food vial where we happened to find three pupae, all of which eventually eclosed.

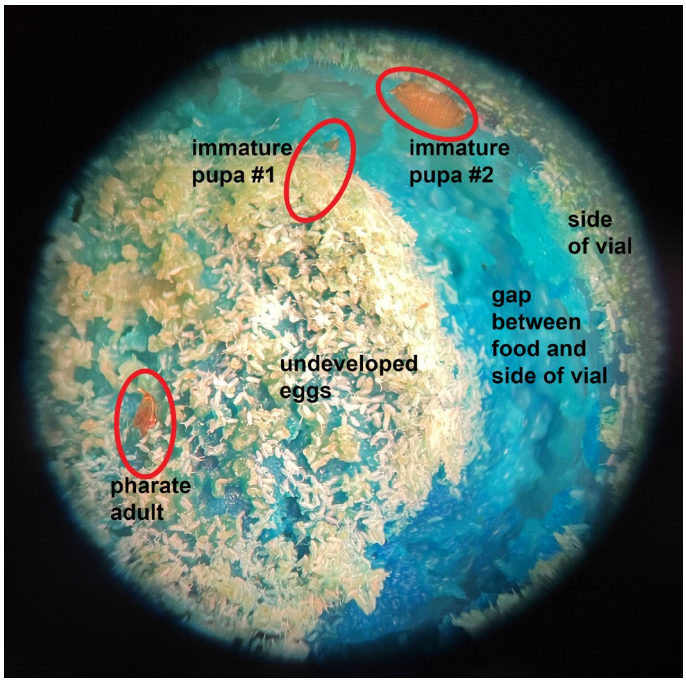

Figure 1. Food surface inside a vial, three of whose eggs (among hundreds) succeeded in developing to the pupal stage (ovals). One of them has reached the pharate adult stage, as indicated by its red eyes. (All three eventually eclosed.) Twenty sons from *tudor*^{1} females were added to this vial's cohort of 50 *pMD*/*TM6B* females 3 weeks earlier (histogram bar in lower left of Figure 2). The maximum number of F_1 offspring from any of the 65 cohorts of unmated virgins was four, so this higher yield implies that spermless males enhance parthenogenesis. A week elapsed since water was added to this vial (see Materials and Methods), so the food shrank from the walls.

To assess the effect of spermless males on parthenogenic output, we crossed homozygous *tudor*^{1} *bw*^{1} *speck*^{1} virgin females (#1786 stock) with Oregon R males and verified the supposedly spermless status of 40 of their sons by adding them (20/vial) to vials containing virgin Oregon R females (50/vial). In no case were any F_1 larvae detected after a week, thus proving the spermless condition of the flies. Finally, we aspirated these (bona fide spermless) males from the first set of vials into a second set (20/vial) with one-week-old virgin *pMD*/*TM6B* females (50/vial) and checked for the presence of F_1 adults.

Figure 2 plots the frequency of F_1 adults (pharate or eclosed) obtained from these two cohorts of females mated with spermless males, compared with the frequency for the other 65 cohorts that remained unmated. The small sample size of this pilot experiment prevents any firm conclusions, but the histograms suggest that spermless males from *tudor*^{1} mothers can indeed enhance the yield of viable F_1 offspring by a factor of two or three.

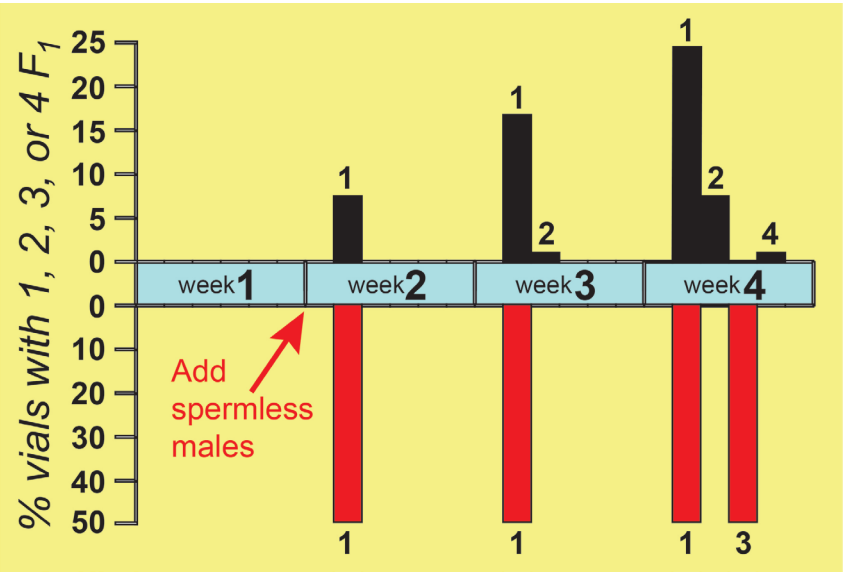

Figure 2. Frequency of vials that yielded viable F_1 progeny over the course of the first month. (Most of the flies died by the end of the second month.) The *x* axis gives the weeks after each cohort was established by placing 50 mature virgin *pMD*/*TM6B* pupae into the vial, while the *y* axis (heights of histogram bars) plots the percentage of vials with 1, 2, 3, or 4 viable F_1 offspring. Subtracting the sum of these percentages from 100 gives the percent of vials having no viable F_1 progeny (i.e., only dead eggs). Data above (black) are for 65 vials with unmated females, while data below (red) are for two vials whose females were crossed with spermless males at the end of the first week. Note: scales differ above *vs.* below the x axis.

Nearly half (34/72) of the F_1 offspring that we obtained from virgins failed to eclose from their pupal case—a trend that was not reported in the investigation by Sperling *et al*. (2023). When we dissected these dead pharate adults, we found a variety of peculiar phenotypes, which prompted us to try to deduce their etiologies. We soon realized that this kind of brainstorming could enable lab instructors to demonstrate the rules of genetics via a "Sherlock Holmes" mystery-solving approach, like the one we used for cold-induced aneuploids (Held *et al*., 2023). Indeed, some of these phenotypes can be explained by the kinds of nondisjunction, aneuploidy, and mosaicism that Sperling and Glover (2024) uncovered among offspring from virgin females.

This experiment lets students find their own unique "pet" flies ("You never know what you're gonna get" – Forrest Gump), which can garner their interest, tweak their curiosity, and prod them to learn the lore of fly genetics on their own (Hartenstein, 1995; Roote and Prokop, 2013). In general, animal anomalies offer the same kind of attraction (minus any exploitation) as the antiquated "freak shows" of traveling circuses (Held, 2021; Kunhardt and Kunhardt, 1995). The virtues of this pedagogical approach are discussed more thoroughly in our companion paper (Held *et al*., 2023).

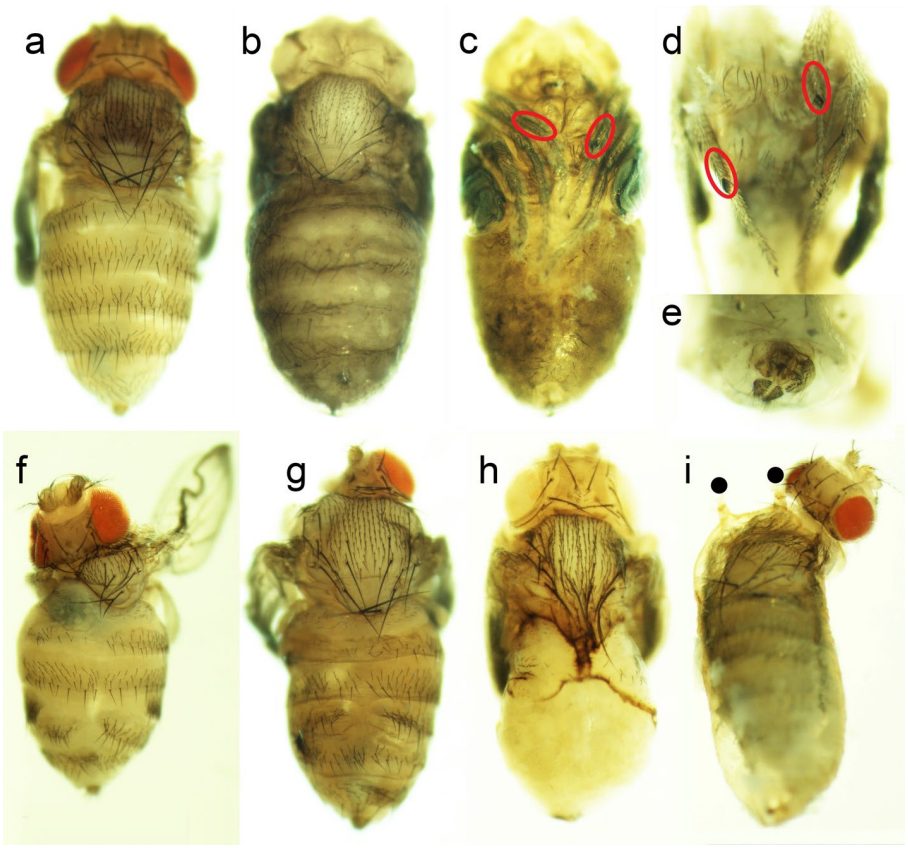

Figure 3. "Rogue's Gallery" of freaky phenotypes (**b-i**), compared with the normal anatomy (**a**) which was exhibited by a majority (22/34) of the dead pharate adults (and eclosed adults) that we recovered from virgin female parents. **b**, **c**. Dorsal (**b**) and ventral (**c**) views of a gynandromorph, whose foreleg basitarsi are encircled with ovals. The leg on the fly's left is male (5 sex comb teeth), while its right leg is female (no sex comb teeth). **d**, **e**. Male with bilateral sex combs (**d**) and slightly rotated male genitalia (**e**). **f**. Fly whose left wing disc (visible as dark tissue beneath the anterior abdominal epidermis) failed to evaginate. Its right wing disc evaginated successfully, but failed to deflate its wing, which remained as an inflated "balloon," instead of flattening to form a proper airfoil. Note the "cleft palate" syndrome of its rear tergites, which failed to fuse properly at the midline (the frontmost cleft being thrice the width of the one behind it). **g**. Fly whose left eye disc failed to develop. There is no obvious evidence of an unevaginated disc on the left side, but we have not yet dissected this specimen to see whether the disc ever developed. **h**. Fly, most of whose abdominal surface lacks any adult epidermis or cuticle. **i**. Fly who evidently did not consult its "instruction manual" for how to exit the pupal case for eclosion. Flies normally use a balloon on the front of their face (the ptilinum) to burst open the flap (operculum) between their anterior spiracles (black dots), but this misguided fly got stuck trying to exit from the side. Another fly that died in its pupal case had only four legs (not shown); it lacked both of its middle legs. (We have not yet dissected it to see if those leg discs are present.) See text for further explanations of the entire assortment of odd phenotypes.

Our "zoo" of F_1 pharate adults (Figure 3) includes a part-male, part-female fly (Figure 3b, c), a four-legged fly (not shown), three flies with half a thorax (Figure 3f), two flies with half a head (Figure 3g), three flies lacking abdominal cuticle (Figure 3h), and one poor little fly that made the fatal mistake of trying to exit its pupal case via the side of its operculum instead of from the front (Figure 3i). To show how deductive logic can be used to tease apart these enigmatic phenotypes, we consider two of the most interesting cases below.

The fly in Figure 3b (dorsal side) and 3c (ventral side) has female genitalia and a female-type right foreleg, but its left foreleg basitarsus is male based on 5 sex comb teeth. This was the only gynandromorph mosaic that we found. Unlike humans, where sex is determined by the presence or absence of a Y chromosome, sex in flies is controlled by the ratio of Xs to the number of sets of autosomes (Cline, 1993), such that 1:2 = male, and 2:2 = female. This individual may have started as an ordinary XX embryo, but then lost an X chromosome from one or more of its nuclei during the syncytial blastoderm stage, leading to a patch of X/0 (male) tissue that eventually wound up in its left forleg. Interestingly, this scenario can also explain why the fly has such thin bristles on its thorax. This thinness could theoretically be due to haploidy, but in that case the density of the bristles should be much higher (Santamaria, 1983). Rather, these thin bristles with normal bristle spacing are more indicative of a haplo-insufficient "Minute" syndrome, which arises when a

gene for a ribosomal protein is lost (Marygold *et al*., 2007). One such gene resides on the tiny 4th chromosome, which could have been lost at the same time as this fly's X chromosome (due to *multiple* nondisjunction errors). The small eyes of this fly are reminiscent of mutations in the *eyeless* gene, which resides on the 4th chromosome, so it is possible that the *pMD/TM6B* stock contains a recessive *eyeless* allele on one of its 4th chromosomes that would only be revealed if the wild-type allele on the other 4th chromosome were lost. If the allele is pervasive in the stock, then half of the flies should have small eyes, but that is not true, so only a subset might bear the allele. This fly also has severely defective abdominal tergites. Because such defects occur commonly among the dead pharate adults, they are more likely to have arisen from developmental mistakes (attributable to parthenogenesis) than to genetic errors.

The fly in Figure 3d (front end) and 3e (rear end) appears to be purely male, based on its sex combs and anatomically normal male genitalia, and it would be natural to conclude that this fly arose in basically the same way as the gynandromorph discussed above, except that the nondisjunction event occurred much earlier (at the first mitosis or during meiosis), leaving the entire fly to develop as a male, and such outcomes have been documented in various parthenogenic *Drosophila* species (Sperling and Glover, 2023). However, the "fly in the ointment" of this conjecture, so to speak, is that the genitalia are rotated by ~30 degrees from the midline (*cf.* Held, 2014 for evo-devo context). Such rotations are common in intersexual flies whose X:autosome ratio is 2:3 (Lindsley and Zimm, 1992). Hence, another variation on the nondisjunction theme may have occurred. Namely, *two* haploid polar bodies might have been recruited back into the haploid starter egg, and if these three sets of chromosomes did not align properly at the first mitosis, then one of the Xs could have accidentally been lost from the composite zygotic nucleus, thus yielding an XX/0 triploid that would manifest an intersexual phenotype. We tested this scenario by inspecting the fly's sex combs, given that intersexes have fewer sex comb teeth. Indeed, we harvested such intersexes as F_2 offspring by mating some of our 3n F_1 females with Oregon R males, and they had ~7 teeth/comb. Our "mystery" fly (Figure 3d) had 10 teeth on the right and 8 on the left, which is slightly more than a 2X:3A intersex, but slightly less than a wild-type male. The evidence in this case is ambiguous.

Collectively, this assortment of bizarre abnormalities recalls the saying "the exception that proves the rule," which is actually a mistranslation from the Latin "*exceptio probat regulam.*" The phrase *really* means "the exception that *probes* (or tests) the rule." The rules in this case are the pathways that govern normal fly development (Held, 2002), and the exceptional flies in this freaky gallery should allow students to better understand how *normal* development works. Indeed, the ability of *any* egg to form a multicellular adult shows that the essence of normal development is the inverse of the motto on our coinage here in the US: *e unum pluribus!*

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References: Ashburner, M., 1989, Drosophila: *A Laboratory Handbook*. CSH Press, Cold Spring Harbor, N.Y.; Boswell, R.E., and A.P. Mahowald 1985, Cell 43: 97-104; Chapman, T., L.A. Herndon, Y. Heifetz, L. Partridge, and M.F. Wolfner 2001. Proc. R. Soc. Lond. B 268: 1647-1654; Craymer, L., 1984, Dros. Inf. Serv. 60: 234; Cline, T.W., 1993, Trends Genet. 9: 385-390; Fankhauser, G., 1945, Q. Rev. Biol. 20: 20-78; Hartenstein, V., 1993. *Atlas of* Drosophila *Development*. CSH Press, Cold Spring Harbor, N.Y.; Hegazy, A.A., A.I. Al-Qtaitat, and R.A. Hegazy 2023, Int. J. Reprod. Biomed. 21: 277-284; Heifetz, Y., U. Tram, and M.E. Wolfner 2001, Proc. R. Soc. Lond. B 268:175-180; Held, L.I., Jr. 1979. W. Roux's Arch. 187: 105-127; Held, L.I., Jr. 1982, Mutat. Res. 94: 87-101; Held, L.I., Jr. 2002, *Imaginal Discs: The Genetic and Cellular Logic of Pattern Formation*. Cambridge Univ. Press, N.Y.; Held, L.I., Jr. 2003. Dros. Inf. Serv. 86: 50-53; Held, L.I., Jr. 2014, *How the Snake Lost Its Legs: Curious Tales from the Frontier of Evo-Devo*. Cambridge Univ. Press, N.Y.; Held, L.I., Jr. 2017, *Deep Homology?: Uncanny Similarities of Humans and Flies Uncovered by Evo-Devo*. Cambridge Univ. Press, N.Y.; Held, L.I., Jr. 2021, *Animal Anomalies: What Abnormal Anatomies Reveal about Normal Development*. Cambridge Univ. Press, N.Y.; Held, L.I., Jr., *et al*. 2023. Dros. Inf. Serv. 106: 15-19; Kalb, J.M., A.J. DiBenedetto, and M.F. Wolfner 1993. Proc. Natl. Acad. Sci. 90: 8093-8097; Kunhardt, P.B., Jr., and P.B. Kunhardt, III 1995, *P.T. Barnum: America's Greatest Showman*. Knopf, N.Y.; Lattao, R., *et al.,* 2011, Fly (Austin) 5: 369-370; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, N.Y.; Marygold, S.J. *et al*., 2007. Genome

Biol. 8: R216; Novitski, E., 1984, Dros. Inf. Serv. 60: 157; Orr, B., K.M. Godek, and D. Compton 2015, Curr. Biol. 25: R538-R542; Roote, J., and A. Prokop 2013, G3: Genes, Genomes, Genetics 3: 353-358; Santamaria, P., 1983, Dev. Biol. 96: 285-295; Simon, J.-C., *et al.,* 2003, Biol. J. Linnean Soc. 79: 151-163; Sperling, A.L., D.K. Fabian, E. Garrison, and D.M. Glover 2023, Curr. Biol. 33: 1-16; Sperling, A.L., and D.M. Glover 2023a, Proc. R. Soc. B 290: 20230261; Sperling, A.L., and D.M. Glover 2023b, STAR Protocols 4: 102585; Sperling, A.L., and D.M. Glover 2024, Heredity 132: 89-97; Thomalla, J.M., and M.F. Wolfner 2023, Curr. Biol. 33: R904-R906; Thomson, T., and P. Lasko 2004. Genesis 40: 164-170; Wakimoto, B.T., D.L. Lindsley, and C. Herrera 2004. Genetics 167: 207-216; Xue, L., and M. Noll 2000, Proc. Natl. Acad. Sci. 97 #7: 3272-3275.

Guide to Authors

Drosophila Information Service prints short research, technique, and teaching articles, descriptions of new mutations, and other material of general interest to *Drosophila* researchers. The current publication schedule for regular issues is annually, with the official publication date being December. The annual issue will include material submitted during the calendar year. The submission deadline for each annual issue will be 31 December of the publication year, but articles are accepted at any time. Submissions should be in MS Word. .

Submission: Proofs will not be sent to authors unless there is some question that needs to be clarified. The editor reserves the right to make minor grammatical, spelling, and stylistic changes if necessary to conform to DIS format and common English usage. Tables may be slightly reformatted to conform to DIS style. Color illustrations now appear in color in both the printed version (from: www.lulu.com) and on our journal web site (www.ou.edu/journals/dis).

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Green, R.L., 1998, Heredity 121: 430-442.
Waters, R.L., J.T. Smith, and R.R. Brown 1990, J. Genet. 47: 123-134.

Note the initials are before each name except for the senior author.

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Drosophila melanogaster: An expedient model for *in vivo* assessment of nanoparticles.

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Emerging Field of Nanobiotechnology

Nanoparticles (NPs) that are characteristically sized between 1-100 nm tremendously benefit human health and lifestyle. NPs have enhanced permeation due to their small size, and the high surface area to volume ratio enables vivid decoration of their surfaces with compounds of interest. Owing to these advantageous features, their presence in consumer goods such as cosmetics, paints and ceramics, surface coatings, and fabrics, is prominent, and their use in medicine for illness prevention, diagnosis, and treatment is increasingly becoming irreplaceable.

Further, in clinical practice, achieving precise control over spatio-temporal distribution of pharmacologically active substances greatly influences its therapeutic benefits. To realise this, designing 'nano-drugs' is an active research niche, residing within the aegis of nanomedicine and nanobiotechnology. The field of nano-medicine combines formulation of the active substances with a nano-particle (NP) based delivery system, to achieve (a) improved therapeutic efficacy, (b) minimize effective drug dosage by increasing its bio-availability, and (c) decrease side effects (Tomé *et al.,* 2021). Nanoparticle-based drug delivery can be used for the treatment of challenging neurological disorders by targeting the drug for the central nervous system (CNS) by crossing the blood-brain barrier (Mittal and Hazari, 2023).

However, prior to their clinical and consumer use, the nano-formulation has to undergo rigorous testing, to assay their toxicity, and its bioaccumulation, bio-uptake, bio-distribution, and bioavailability. Most popularly, *in vitro* testing is conducted to study these parameters and to understand the potential adverse mechanisms induced by the NPs into the biological system. These assessments can effectively identify critical dosages of 'nano-drugs', however, in a cell-specific environment. To precisely assay the biological effects, *in vivo* assessments are crucial as they more accurately examine the relevance and safety of the formulation for human use (Savage *et al.,* 2019).

The use of a mammalian system for *in vivo* testing of NPs is challenged by ethical constraints and high operational cost, whereas use of vertebrate models such as *Danio rerio* is incompatible for high throughput screening of the NPs due to their longer generation time and smaller brood size, compared to invertebrate models. Among the invertebrate model organisms, *Drosophila* model combines the advantages of sharing significant homology to humans, ethically less controversial, and have a smaller generation time, perceptible developmental stages, and high fecundity. Further, a wide array of tools for genetic manipulation of the fly model and phenotypic assays to readout the effect of NP administration are available, making it a good fit for high throughput screening assessment of *in vivo* administration NP (Ong *et al.,* 2015).

Administrating NPs in *Drosophila*

NPs can be designed from different materials and tailored into an array of shapes and sizes, which play a crucial role in their mode of activity and as well as determine its toxicity. Apart from this, successful delivery of NPs and deriving its desirable effect is also majorly dependent on their mode of administration.

Based on the nature of NP formulation, the major route of human exposure to nanoparticles is through inhalation, dermal penetration, ingestion, and injection. Similarly, NPs can be administered in *Drosophila* by either of these methods, and the efficacy of each can be assayed. A nebulizer-based method for nasal administrating NPs in flies was developed by Posgai and group (Posgai *et al.,* 2009) for hassle free administration of NPs, primarily directed at evaluating their cytotoxicity. The method is, however, easily

amenable to experimental modification. Oral and injectable routes are beneficial for checking the dose dependent effect of NPs. Different doses of nanoparticles can be easily supplemented to the food, or administered directly in the hemolymph, using microinjection-based techniques. The ease of each of these methods makes the fly model a very useful tool in evaluating the physiological impact of administrating the nano formulation via the different modes.

Assaying effect of NPs in *Drosophila*

The biological effects of NPs on behavioral aspects, fertility, lifespan, and energetics can be easily recorded at different developmental stages and with aging of the adult flies. Researchers have exposed larvae for early and adults for late developmental stages to different types of nanoparticles and examined how the exposure influences locomotor activity, feeding behavior, reproductive success, and overall physiology. The goal is to gain insights into their systemic compatibility, distribution, and the risks associated with nanoparticle exposure. The findings can be relevant by extrapolating the impact noted on fruit flies to the potential implications for other organisms, including humans. The assays to study the impact of nanoparticles in *Drosophila* are briefly provided below.

1. **Development and survival assessment**
   1.1. **Observation of Development Milestones:** Regularly monitor and record the developmental milestones like egg hatching, larval moulting and pupation, and the time taken to reach each stage in larvae or adults raised in the food supplemented or not supplemented with nanoparticles (NP). Any noted difference can be correlated as a consequence of experimental administration of NP.
   1.2. **Lifespan:** For studying lifespan of the flies, freshly eclosed flies can be collected in groups of 20, from each condition, and subsequently transferred into fresh culture conditions every alternate day. The numbers of dead flies during each transfer is recorded, to note the effect of NP on fly survivability (Linford *et al.,* 2013).

2. **Behavioral assessments**
   2.1**. Locomotor Activity**: The locomotor activity in *Drosophila* larva and adults can be monitored as follows. Changes in movement patterns may indicate neurological or physiological effects as an impact of being augmented with the nanoparticle to be tested.
   i. **Larval crawling ability** can be monitored by making them crawl in a groove engraved on agar plate. To determine the locomotor activity the distance covered by each larva in 30 seconds placed in a groove has to be recorded (Raj *et al.,* 2017b).
   ii. For **adult** flies, climbing and flying ability can be evaluated. **Climbing ability** is recorded as the number of flies that vertically ascend and cross a set mark in a glass tube, within a stipulated time. To begin with, ten flies per condition are first allowed to acclimatize within the experimental set up, followed by gently tapping them to the bottom of the glass tube and then permitting them to ascend (Raj *et al.,* 2017b). **Flying ability** of flies can be assessed by setting up an experiment where a vacant vial is positioned at the centre of a 14.5 cm diameter beaker, filled halfway with water to establish a barrier between the fly-containing vial and the beaker's wall. The inner surface of the beaker is coated with yeast paste to provide a stimulus for flying to the pre-starved flies. Subsequently, this vial-in-beaker setup will be placed within a larger enclosure with a 23 cm diameter for a duration of 40 minutes. To assess flight ability, flies released from the vial, and those that successfully flew, traversed the water barrier, and landed on the walls of the beaker or enclosure have to be designated as flyers. On the other hand, flies that remained within the vial or succumbed to drowning in the water surrounding the vial will be categorized as non-flyers. (*RajEtal(Pg18).Pdf*, n.d.).

   2.2**. Feeding Behavior**: Examine feeding habits to assess the influence of NPs on nutrient uptake and metabolism. Methods popularly used to check feeding behavior in *Drosophila* are -

i. **Food Coloring dyes**: A non-toxic dye (such as food colors) can be mixed with yeast to make a colored food paste. Allow the larva/flies to feed on colored food for a definite period and thereafter homogenize, and quantify the food intake by correlating with the spectrophotometric quantification of dye ingested (Aditi *et al.,* 2016).

ii. **Proboscis Extension Reflex (PER) Assay**: This method involves recording the number of times the fly extends its proboscis (feeding tube) in response to the presence of food, as an indicator of the fly's feeding response (Shiraiwa & Carlson, 2007).

iii**. Capillary Feeder (CAFE) Assay**: The CAFE assay involves providing flies with access to liquid food through a capillary tube. Flies are housed in vials with a capillary tube containing a liquid food source. Changes in the volume of the liquid over time gives an assessment of fly's feeding behavior (Diegelmann *et al.,* 2017).

3. **Physiological Assessments**: Performed to check the effect of NPs on overall physiology of the fly, by assaying following parameter, in cell or tissue level.

3.1. **Metabolic Homeostasis:** Assess the metabolic aspects which includes nutrient uptake, energy balance, metabolic pathways and transcriptional analysis of genes involved. Any alteration of these parameters is suggestive of a disruptive metabolic homeostasis. Some of the common assays used to study metabolic aspects –

i. **Body size and weight:** The difference in body weight and size can be easily observed as a preliminary indicator of any change in the metabolic activity of fly (Agrawal & Shah, 2020).

ii. **Carbohydrate estimation:** To further evaluate if indeed there is any metabolic alteration in the flies due to NP administration, the levels of metabolic moieties like glycogen and trehalose can be estimated by the most common anthrone-sulfuric acid assay which measures the glucose released from glycogen and trehalose during hydrolysis step (Raj *et al.,* 2017b).

iii. **Protein estimation:** To estimate the protein content, multiple methods can be used like Lowry, bicinchoninic acid assay and the most common being the Bradford assay in which the protein content has to be calculated based on absorbance readings and standard curve (Tennessen *et al.,* 2014).

iv. **Lipid estimation:** To estimate the amount of lipid, the samples were dried to obtain dry weight and later the ether soluble lipids were extracted from samples so that the lipid content can be calculated by subtracting lipid free weight of flies from dry weight (Aditi *et al.,* 2016).

v. **Lipid droplet staining**: The most common method used is Nile red staining. It is a fluorescent dye that becomes strongly fluorescent in a hydrophobic environment such as lipid droplets. It is a sensitive method to visualize lipid droplets and for qualitative as well as quantitative analysis of lipid (Aditi *et al.,* 2022)**.**

vi. **Quantitative Real –Time PCR (qPCR):** Analyse changes in expression of genes related to metabolism using qPCR to assess impact of NPs in metabolic pathways using SYBR Green dye or Taqman probe assay (Singh and Agrawal, 2021).

3.2. **Oxidative Stress:** Measure oxidative stress marker like reactive oxygen species to evaluate cellular damage caused by NPs. Commonly used methods include –

i. **Dihydroethidium (DHE) Staining:** DHE is a fluorescent dye that becomes oxidized by superoxide radicals and emits red fluorescence. Incubate *Drosophila* tissues or whole flies with DHE and then visualize the fluorescence using fluorescence microscopy. Higher fluorescence indicates higher levels of superoxide ions (Aditi *et al.,* 2022).

ii. **Hydrogen Peroxide (H_2O_2) Assay:** A colorimetric or fluorometric assay to quantify hydrogen peroxide levels can be used. The assay typically involves the reaction of H_2O_2 with a substrate that produces a detectable signal (Barata and Dick, 2013).

3.3. **Genotoxicity:** To assess DNA damage by NPs that can have potential genetic effects, common methods used are –

i. **Comet Assay (Single Cell Gel Electrophoresis):** The comet assay detects DNA damage at the level of individual cells. Isolate cells from *Drosophila* tissues and embed them in agarose gel. Subject the gel to electrophoresis, causing damaged DNA to migrate away from the nucleus, creating a "comet" tail. The extent of tail formation is quantified, to assay the DNA damage (Gaivão and Sierra, 2014).

ii. **Mutation Detection Assays:** Use specific genetic markers or reporter genes that are sensitive to mutagenic events. Examples include the white/scarlet eye color marker or mutations in specific genes associated with eye or wing development. The frequency of mutant phenotypes in the offspring of flies fed with NPs is then measured.

iii. **Somatic Mutation and Recombination Test (SMART):** The SMART assay involves the use of *Drosophila* wing cells and can detect somatic mutations and recombination events induced by genotoxic agents. The frequency of mutant spots on the adult wings can then be evaluated (Gunes *et al.,* 2021).

3.4. **Pigmentation Defects:** The effect of NPs can also be seen on body color. The pigmentation changes at pupal and adult stages can be noted – a parameter to assay effect of NP on melanin synthesis (Agrawal and Shah, 2020).

4. **Reproductive Success**: The assay investigates the effect of NP administration on fertility, egg laying, and overall reproductive success (Singh *et al.,* 2021). Assays and techniques commonly used for studying reproductive health in *Drosophila* are –

   i. **Ovary Morphology:** Dissect out ovaries from females and examine them under a microscope. The developmental stage of the oocytes (egg cells), their size and the overall health of the ovaries can be evaluated (Raj *et al.,* 2017a).

   ii. **Fecundity Assay:** Quantify the number of eggs laid by female flies placed individually in egg collection medium, over a specified period (Ng'oma *et al.,* 2018).

   iii. **Egg Viability Assay:** Assess the hatching rate of eggs laid by females. Eggs are collected on suitable medium, and the number of larvae that successfully hatch are counted (Raj *et al.,* 2017a).

   iv. **Courtship and Mating Assays:** Observe courtship behaviors amongst males and females. Record the time taken for mating to occur between flies reared on control and NP supplemented food. The copulation duration and frequency can be evaluated (Koemans *et al.,* 2017).

5. **Transgenerational Effects:** To assays the impact of NPs on subsequent generations, the aforementioned parameters can be assayed in the progeny for several generations to come without any exposure to NPs (Jimenez-Guri *et al.,* 2021). Since *Drosophila* generation time is only 10-12 days at ambient culture condition, the effect across multiple generations can be quickly screened, to check for any adverse effect of the NPs, if fed to parents and never to the progeny.

Conclusion

Drosophila melanogaster stands out as a valuable model organism for studying the effect of NP due to its short lifespan, cost effectiveness, genetic relatedness to humans, and ease of performing multiple assays to check various behavioral, physiological, and molecular aspects. Nanotechnology has come out to be a boon for the healthcare industry offering various methods for diagnosis and treatment. The utility of *Drosophila* in nanoparticle research not only facilitates a deeper understanding of the potential risks associated with nanoparticle exposure but also to the development of safer nanomaterials through informed and targeted experimentation. This note aims at how *Drosophila* can be convincingly used for the *in vivo* assessment of NPs using the different techniques.

References: Aditi, K., M.N. Shakarad, and N. Agrawal 2016, Scientific Reports 6: 31411; Aditi, K., A. Singh, M.N. Shakarad, and N. Agrawal 2022, Experimental Biology and Medicine (Maywood, N.J.) 247(2); Agrawal, N., and P. Shah 2020, Toxicology of Nanoparticles: Insights from *Drosophila*; Barata, A.G., and T.P. Dick 2013, Methods in Enzymology 526: 61–82; Diegelmann, S., A. Jansen, S. Jois, K. Kastenholz, L. Velo Escarcena, N. Strudthoff, and H. Scholz 2017, Journal of Visualized Experiments, JoVE

121: 55024; Gaivão, I., and L.M. Sierra 2014, Frontiers in Genetics 5: 304; Gunes, M., K. Ates, B. Yalcin, S. Akkurt, S. Ozen, and B. Kaya 2021, Electromagnetic Biology and Medicine 40(2): 254–263; Jimenez-Guri, E., K.E. Roberts, F.C. García, M. Tourmente, B. Longdon, and B.J. Godley 2021, PeerJ, 9: e11369; Koemans, T.S., C. Oppitz, R.A.T. Donders, and H. van Bokhoven, A. Schenck, K. Keleman, and J.M. Kramer 2017, Journal of Visualized Experiments, JoVE 124: 55808; Linford, N.J., C. Bilgir, J. Ro, and S.D. Pletcher 2013, JoVE 71: 50068; P. Mittal, and P.P. Hazari 2023, Fiber and Textile Engineering in Drug Delivery Systems pp. 417–441; Ng'oma, E., E.G. King, and K.M. Middleton 2018, Fly 12(3–4): 183–190; Ong, C., L.-Y.L. Yung, Y. Cai, B.-H. Bay, and G.-H. Baeg 2015, Nanotoxicology 9(3): 396–403; Posgai, R., M. Ahamed, S.M. Hussain, J.J. Rowe, and M.G. Nielsen 2009, The Science of the Total Environment 408(2): 439–443; Raj, A., P. Shah, and N. Agrawal 2017, PLOS ONE 12(5): e0178051; Raj, A., P. Shah, and N. Agrawal 2017b, Scientific Reports 7(1): 15617; RajEtal(pg18).pdf. (n.d.). Retrieved 31 December 2023; Savage, D.T., Hilt, J.Z., and T.D. Dziubla 2019, Methods in Molecular Biology (Clifton, N.J.) 1894: 1–29; Shiraiwa, T., and J.R. Carlson 2007, Journal of Visualized Experiments, JoVE 3: 193; Singh, A., and N. Agrawal 2021, Molecular Basis of Disease 1867(7): 166127; Singh, A., A. Raj, A. Padmanabhan, P. Shah, and N. Agrawal 2021, Journal of Applied Toxicology, JAT 41(8): 1188–1199; Tennessen, J.M., W.E. Barry, J. Cox, and C.S. Thummel 2014, Methods (San Diego, Calif.) 68(1): 105–115; Tomé, I., V. Francisco, H. Fernandes, and L. Ferreira 2021, APL Bioengineering 5(3): 031511.

Determination of median lethal concentration (LC_{50}) for arsenic to fruit fly, *Drosophila ananassae.*

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Introduction

Heavy metals are naturally occurring, high density elements present in earth crust. These are the elements, which even in trace amount act as environment toxicants causing wide range of health effects on humans (Mitra *et al.*, 2022). Arsenic is also one of heavy metals from toxicology perspective. It is an omnipresent metalloid and 20th most abundant constituent element of earth crust (Ahmad *et al*., 2020). Now, arsenic has been ranked at top of the priority list of hazardous substances in ASTDR and despite being less than many heavy metals, arsenic held this position based on its frequency and potential for human exposure (Wu *et al*., 2021; Islam *et al*., 2022). Its contamination recently received worldwide attention as it is a major threat to public health in more than 108 countries including India (Shaji *et al*., 2021). Arsenic poisoning in India is more than 50 µg/L covering almost 20 states becoming the biggest natural groundwater calamity to human beings (Shukla *et al*., 2020). According to reports, more than 200 million people around the world are suffering from arsenic contamination (Rahaman *et al*., 2021). The contamination sources of arsenic can be both natural and anthropogenic. Natural sources include weathering of rock and volcanic eruptions, while herbicides, mining of ores, smelting of Cu, Pb, NI, and wood preservatives include anthropogenic sources (Garelick *et al*., 2008). Human exposure to arsenic is mainly via contaminated food and water (Rahaman *et al*., 2021). Chronic arsenic exposure is associated with increased risk of liver, bladder, and skin cancer, cardiovascular diseases, diabetes mellitus, neuropathy, and ocular diseases (Mazumder, 2008).

Drosophila flies have been preferred to investigate the impact of various edible and toxic substances on their genetical, developmental, behavioral and molecular phenomena. These flies are sensitive indicators of environmental contaminants that may disrupt their normal physiology, behavior and may cause genetic mutation. With respect to genetic similarity, *Drosophila* is one of the closest invertebrate model organisms to humans. Nearly 60% genome in humans resembles that of *Drosophila* and 75% of genes related with disease in humans have homolog in flies (Fortini *et al*., 2000). Besides this, many genes essential for reproduction are conserved in *Drosophila* and vertebrates. *Drosophila* is an excellent *in vivo* genetic model organism for studying toxicity related aspects like consequences of heavy metals on its life history traits. *D. ananassae* is a cosmopolitan species that is mainly prevalent in the close proximity to human habitation. This species of *Drosophila* possesses several unique genetical characteristics that make it different from other species of this genus (Singh, 2010). One such peculiar characteristic is spontaneous male meiotic crossing over that does not occur in other members of *Drosophila* (Singh and Singh,1987, 1988). In this research note, we have determined the lethal concentration of arsenic at which 50 percent of the flies of both sexes of *D. ananassae* die. Sex-wise analysis of LC50 helps to evaluate whether the two sexes show variation in the tolerance of arsenic toxicity.

Materials and Methods

D. ananassae flies were collected from Varanasi by using banana bait. These flies were first reared for 5 generations in the laboratory on culture medium containing maize powder, agar-agar, yeast, brown sugar, propionic acid and nipagin (antifungal chemical) at constant laboratory temperature (24 ± 1°C) keeping 12:12 light and dark condition. The experiments pertaining to LC50 determination of sodium arsenite were performed on these flies. Sodium arsenite (NaAsO2) chemical used in the experiments was purchased from loba chemie (product code 0577600500). Food used for the experiments contained varying amount of sodium arsenite and, therefore, in the prepared food, this chemical was added in to make food concentration of 0.1 mM, 0.25 mM, 0.50 mM, 0.75 mM, 1 mM, 1.5 mM, 2 mM and 3 mM. This amended food was then poured into glass vials up to nearly 2.5 cm of height (vial height 8.0 cm × diameter 4.0 cm) and allowed to solidify completely. In each vial, 7 days old 10 virgin flies (females and males separately) were transferred. Hence, 30 flies of a particular sex were used for each concentration to study the lethal effect of sodium arsenite. Similarly, 30 flies were also studied as control reared in the vials containing normal composition of food (without arsenic). The entire experiments were performed twice. Further, if mortality of fly occurred due to its adherence to the food or any other kind of stress instead of arsenic toxicity, then such cases were not counted. Flies on each medium were observed for 4 days (96 h), and after every 24 h, the number of flies dead in each vial was recorded. The mortality

percentage after 4 days for both female and male flies was calculated, and data were analysed by log-probit method using SPSS software for LC50 determination.

Results and Discussion

Flies reared on normal food did not show mortality during four days of observation (24 h, 48 h, 72 h, and 96 h), whereas, flies cultured on the food containing arsenic showed mortality. Tables 1 and 3 show the mortality of both female and male flies, respectively, observed for all 8 concentrations each day till 96 h. The data were analysed by log-probit method suggested by Finney using SPSS software. The Tables 2 and 4 and graphs generated by software are shown in Figures 1 and 2 for female and male, respectively. It was seen that mortality for both male and female flies increased significantly in time and dose dependent manner. The LC50 for female flies from probit table with confidence limit 95% was found to be 0.39 mM and for males, it was 0.23 mM. The present observation also indicates that male flies were more sensitive to arsenic than females.

Table 1. Mortality rate of female flies of *D. ananassae* treated with different concentration of NaAsO2.

Conc. (mM)	No. of dead female flies at varying time periods				Total mortality
	24h	48h	72h	96h	
0.1	0	0	0	0	0
0.25	0	2	3	3	8
0.5	2	3	7	8	20
0.75	4	4	7	9	26
1	4	6	9	7	28
1.5	4	7	7	10	28
2	5	6	8	8	29
3	5	7	12	6	30

Arsenic is a toxic metalloid that can be harmful to animals, including humans, when present in excessive amounts. Animals can be exposed to arsenic through various sources such as contaminated water, soil, plants, and feed. Its toxicity can occur through both acute and chronic exposure. Arsenic has been a matter of concern and major risk factor for public health all over the world. Due to its global prevalence, toxicity and carcinogenicity, it is considered as one of the most hazardous chemicals on our planet (Shanmugapriya *et al*., 2015; Rahaman *et al*., 2021). In this study, we determined LC50 for both male and female flies for arsenic (NaAsO2). It was found that there was a significant difference in the concentration at which both flies were showing 50% mortality. Males were found to show 50% mortality at much lower concentration than females. Even 100% mortality rate for male flies was found to be at much lower concentration than for females. The concentration at which male flies

Table 2. Probit table of female mortality for NaAsO2 generated by SPSS.

Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(conc)[a]		
	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
0.010	.076	.041	.112	-1.121	-1.382	-.951
0.020	.092	.053	.131	-1.037	-1.275	-.881
0.030	.104	.062	.146	-.984	-1.208	-.837
0.040	.114	.070	.157	-.944	-1.157	-.803
0.050	.123	.077	.168	-.911	-1.115	-.776
0.060	.131	.083	.177	-.884	-1.080	-.753
0.070	.138	.089	.185	-.859	-1.050	-.732
0.080	.145	.095	.193	-.837	-1.022	-.714
0.090	.152	.101	.201	-.818	-.997	-.697
0.100	.159	.106	.208	-.799	-.974	-.682
0.150	.189	.132	.241	-.724	-.880	-.617
0.200	.217	.157	.272	-.664	-.805	-.566
0.250	.244	.181	.301	-.613	-.742	-.521
0.300	.271	.207	.331	-.566	-.685	-.480
0.350	.299	.233	.362	-.524	-.633	-.442
0.400	.329	.260	.394	-.483	-.585	-.405
0.450	.360	.290	.428	-.444	-.538	-.368
0.500	.393	.321	.466	-.405	-.493	-.332
0.900	.975	.808	1.254	-.011	-.093	.098
0.990	2.045	1.534	3.142	.311	.186	.497

Logarithm base = 10

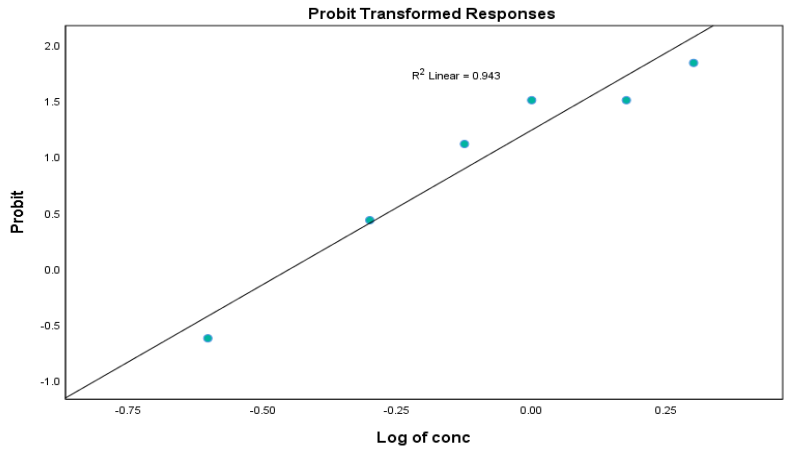

Figure 1. Probit transformed graph for the female flies of *D. ananassae.*

Figure 2. Probit transformed graph for the male flies of *D. ananassae.*

Table 3. Mortality rate of male flies of *D. ananassae* treated with different concentration of $NaAsO_2$

Conc. (mM)	No. of dead male flies at varying time period				Total mortality
	24h	48h	72h	96h	
0.1	0	0	1	1	2
0.25	0	0	11	4	15
0.5	0	2	10	10	22
0.75	0	5	11	14	30
1	0	2	14	14	30
1.5	2	9	13	6	30
2	2	11	14	3	30
3	4	10	10	6	30

Table 4. Probit table for male mortality exposed to $NaAsO_2$ (generated by SPSS).

95% Confidence Limits for conc.				95% Confidence Limits for log(conc.)[a]		
Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
0.010	.065	.038	.093	-1.184	-1.425	-1.031
0.020	.077	.046	.107	-1.113	-1.333	-.973
0.030	.085	.053	.116	-1.069	-1.275	-.935
0.040	.092	.059	.124	-1.035	-1.232	-.907
0.050	.098	.064	.130	-1.007	-1.197	-.885
0.060	.104	.068	.136	-.984	-1.167	-.865
0.070	.109	.072	.142	-.964	-1.140	-.848
0.080	.113	.076	.147	-.945	-1.117	-.832
0.090	.118	.080	.152	-.929	-1.095	-.818
0.100	.122	.084	.157	-.913	-1.076	-.805
0.150	.141	.101	.177	-.850	-.995	-.751
0.200	.159	.117	.196	-.799	-.932	-.708
0.250	.175	.133	.214	-.756	-.878	-.670
0.300	.192	.148	.232	-.717	-.829	-.635
0.350	.208	.164	.250	-.681	-.786	-.603
0.400	.225	.180	.268	-.647	-.744	-.571
0.450	.243	.197	.288	-.614	-.705	-.540
0.500	.262	.215	.310	-.581	-.667	-.509
0.900	.563	.469	.721	-.249	-.329	-.142
0.990	1.050	.804	1.580	.021	-.095	.199

showed complete mortality was at 0.75 mM and for female, it was 3mM. Data obtained in this regard showed that males were more sensitive towards NaAsO2 than were females. One of the possible explanations for apparent difference in the toxicity in the two sexes could be due to variation in their size and fat content present in them. Females are larger in size and possess more fat bodies, which is primarily responsible for the detoxification of chemicals in flies, so may be more fat bodies provide a sort of insulation against this toxicant (Nadda *et al*., 2005). Males being smaller in size and containing lesser amount of fat body may be more susceptible towards its toxicity. Further, more detailed experiments are therefore required to be done to investigate this aspect. This brief preliminary study helps us to suggest the specific LC50 concentration of arsenic for the flies of *D. ananassae* and to decide the optimum doses of it that do not cause flies mortality at all and can be used to study various life history aspects of *D. ananassae*.

Acknowledgment: SU is thankful to her lab mate, Ms. Ankita Das for assistance and discussion during the analysis of the data. Financial support in the form of UGC-JRF to SU and IoE (Institute of Eminence) seed and incentive grant, Banaras Hindu University, to AKS is thankfully acknowledged.

References: Ahmad, H.I., M.B.B. Majeed, A. Jabbar, R. Arif, and G. Afzal 2020, Reproductive Toxicity of Arsenic: What We Know and What We Need to Know? *In:* (Ed.), *Environmental Health*. Intech Open; Fortini, M.E., M.P. Skupski, M.S. Boguski, and I.K. Hariharan 2000, The Journal of Cell Biology150: 23-30; Garelick, H., H. Jones, A. Dybowska, and E. Valsami-Jones 2008, *Reviews of environmental contamination and toxicology*197: 17–60; Mazumder, D.N.G., 2008, The Indian journal of medical research 128: 436–447; Islam, R., L. Zhao, Y. Wang, G. Lu-Yao, and L.Z. Liu 2022, Cancers 14: 4502; Mitra, S., A. Chakraborty, A. Tareq, T. Emran, F. Nainu, A. Khusro, A. Idris, M. Khandaker, H. Osman, F. Alhumaydhi, G. Simal, and Jesus 2022, Journal of King Saud University – Science 34: 101865. 10.1016; Nadda, G., P.N. Saxena, and G. Srivastava 2005, Applied Entomology and Zoology 40: 265-271; Rahaman, M.S., M.M. Rahman, N. Mise, M.T. Sikder, G. Ichihara, M.K. Uddin, and S. Ichihara 2021, Environmental Pollution 289: 117940; Shanmugapriya, S.P., J. Rohan, and D. Alagiyameenal 2015, Journal of Chemical and Pharmaceutical Research 7(10S): 174-177; Shukla, A., S. Awasthi, R. Chauhan, and S. Srivastava 2020, Arsenic in drinking water and food, 1-12. DOI:10.1007/978-981-13-8587-2_1; Singh, B.N., 2010, Indian journal of experimental biology 48: 333–345; Singh, B.N., and A.K. Singh 1987, Genome 29: 802-805; Singh, A.K., and B.N. Singh 1988, Genome 30: 445-450; Wu, Y., R. Ding, X. Zhang, J. Zhang, Q. Huang, L. Liu, and H. Shen 2021, Environment international 147: 106360.

Probing a novel structure in the germarium of the *Drosophila* ovary.

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*(This work was initiated in the laboratories of Dr. Terry Orr-Weaver and Dr. David Page, and currently in the laboratory of Dr. Ruth Lehmann.).

Abstract

During a detailed ultrastructural study of the germarium in the *Drosophila* ovary, an unusual structure was found containing microrods, 10 nm × 1 μm in length with a central hole of 2-3 nm. Further analysis indicated that these structures are only found in older flies and are restricted to a novel organelle consisting of two or more adjacent endoplasmic reticula with either microrods or amorphous electron density between adjacent ER membranes. Although the nature of these microrods is not known, they are remarkably similar to the molecular structure of viral dsRNA, determined by crystallography. If this can be verified, it would indicate that the fly germline assembles dsRNA into a new organelle in the anterior portion of the germarium.

Introduction

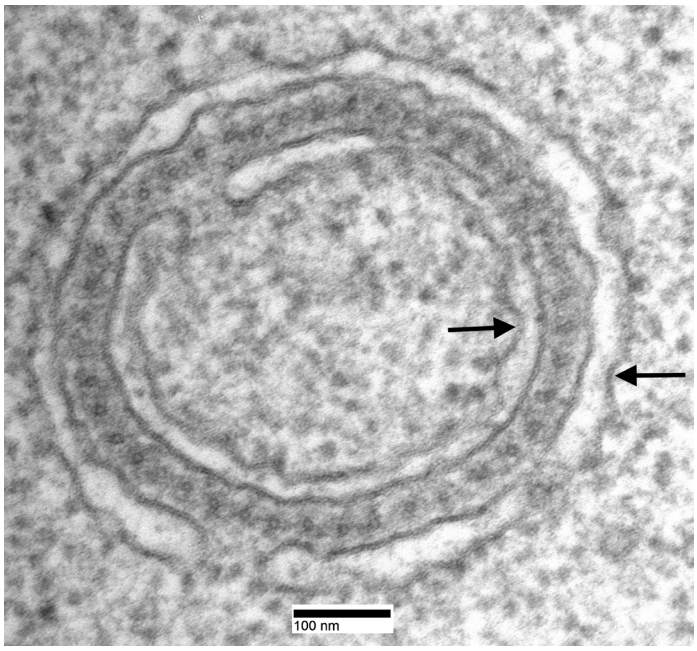
The *Drosophila* ovary consists of a number of individual ovarioles, each composed of a germarium at the anterior tip followed by 14 stages of oocyte development (King, 1970). At the anterior tip of the germarium 2 to 3 germline stem cells are located; they divide and produce another stem cell and a cystoblast that will divide four times to produce a cluster of 16 inter-connected cells, one of which will become the oocyte and the other 15 nurse cells. The cluster of 16 cells becomes surrounded by follicle cells after the fourth division. After the completion of meiosis and the completion of the follicular layer (called stage 1), development continues through stages 2 to 14 within the ovariole to complete oogenesis (King, 1970). Many detailed studies of the germarium have been published, including some from my laboratory (Mahowald and Strassman, 1970; Tazuke *et al*., 2002), but none have described the unusual endoplasmic reticulum (ER) structures described in this article. As far as I know, these structures are not described in any other studies of the *Drosophila* ovary.

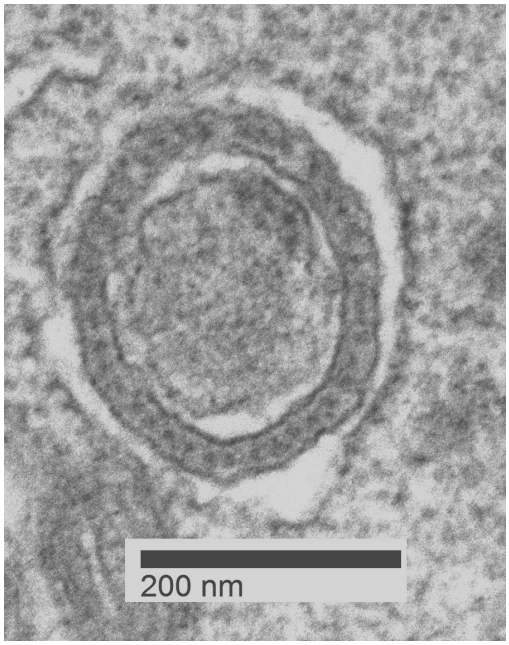
Novel Structures in Aged Female Flies

The initial structure, found in Region 2A of the germarium of a 3 week old fly obtained from a large population cage in T. Orr-Weaver's laboratory, was discovered in thin sections prepared for electron microscopy. An organelle, less than a half micron in diameter (Figure 1a), is outlined by two sets of endoplasmic reticulum (ER) membranes. Between the ERs there are 44 circular structures, 10 nm in diameter with a 2-3 nm central hole. Serial sections demonstrate that the structure is actually a cylinder, more than 1 μ in length, and tapered at each end. This indicates that the circular structures are actually rods existing between two adjacent ER membranes. A similar structure was found in another laboratory stock (Figure 1b). Finally, to determine whether these structures could be duplicated, I set up a small population of wild type flies in a bottle, sealed by a mesh instead of a cotton plug, to mimic a population cage environment. I again detected similar structures between adjacent ER membranes (Figure 1c). Since there are no previous descriptions of rods of this size, I will name them "microrods".

There are other structures discovered in the flies from Orr-Weaver's population cage that displayed similar properties of electron density between two adjacent ER membranes. One consists of sheets of two or more adjacent ER membranes, with electron density appearing between adjacent ER membranes (Figure 2). From the analysis of 10 serial sections (each 0.2 μ in thickness) this organelle was adjacent to the nucleus and more than 1.5 μ × 2 μ in size. In another example, a triple row of ER membranes had two electron densities between alternating ER membranes (Figure 3). In the upper part of the figure, the plane of section through the

two densities provides a tangential image of microrods (arrow). Measurement of the thickness of the "rod" and the spacing corresponds to the measurements of microrods in Figure 1.





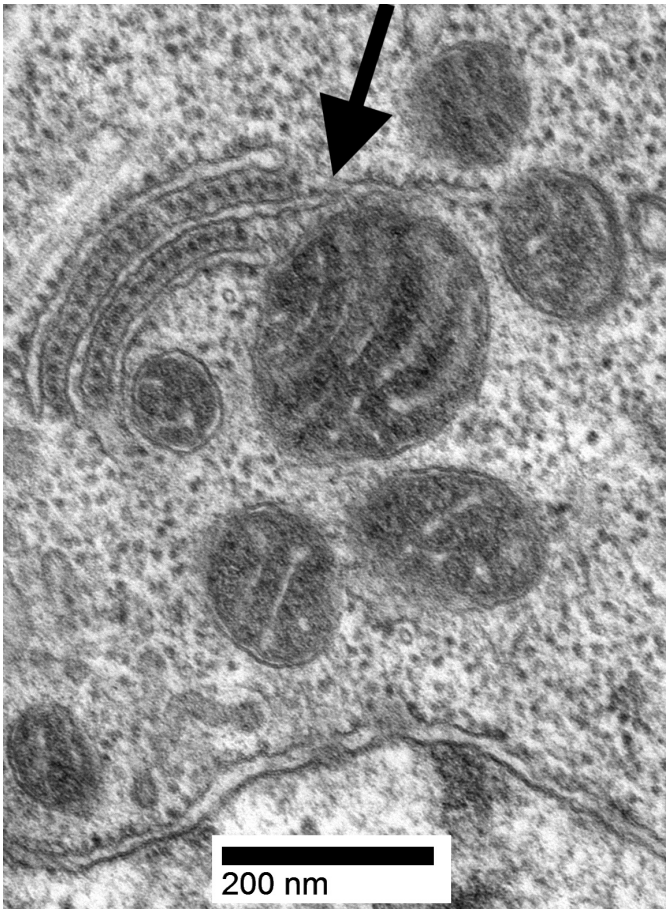


Figure 1a, above left. Cross-section of a 0.5 µ cylinder (seen through 11 serial sections) composed of two sets of ER membranes (arrows) between which are found 44 µ pods, 10 nm in diameter with a central hole of 2-3 nm. The outer ER membrane has some ribosomes attached (hence rER). This structure was found in old flies obtained from a large population cage.

Figure 1b, above right. Cross-section of another cylinder, with 35 – 40 microrods, located between two adjacent ER membranes. This structure was found in a laboratory stock of flies (age of the flies is unknown).

Figure 1c, left. Two rows of microrods between adjacent ER membranes (cf. arrow) in region 2a of an OrR fly germarium.

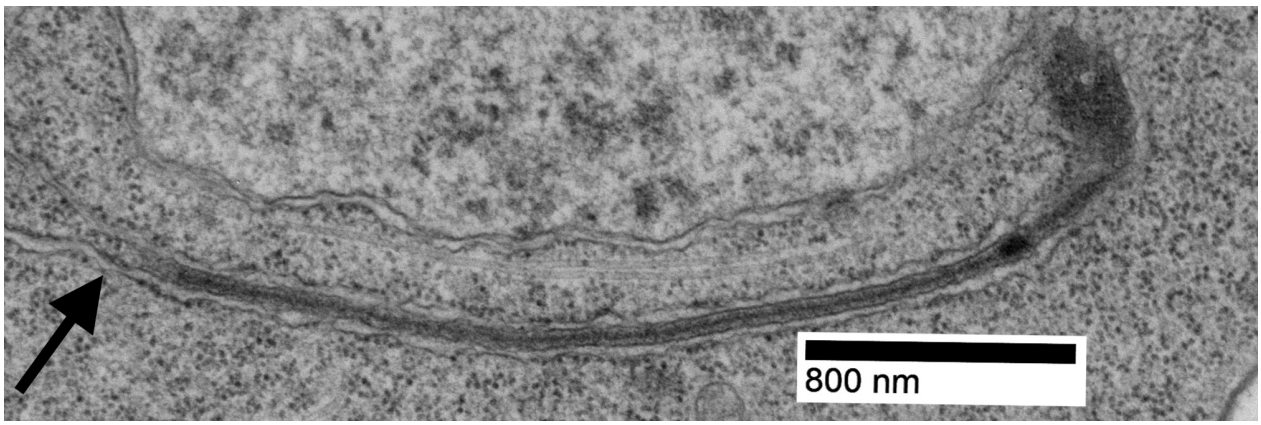


Figure 2. Cross-section through a 3 μ square, sheet like organelle (determined from a set of 12 serial sections), located adjacent to the nucleus of a cystocyte in region 2a. The organelle consists of two adjacent ER organelles with a layer of density between the two inner membranes. The outer membrane is marked by many ribosomes. The arrow points out that the membrane layers in this organelle are continuous with two rER membranes. The central density appears to be organizing into microrods (note the periodic density, arrow). Note the microtubules that are parallel to this structure.

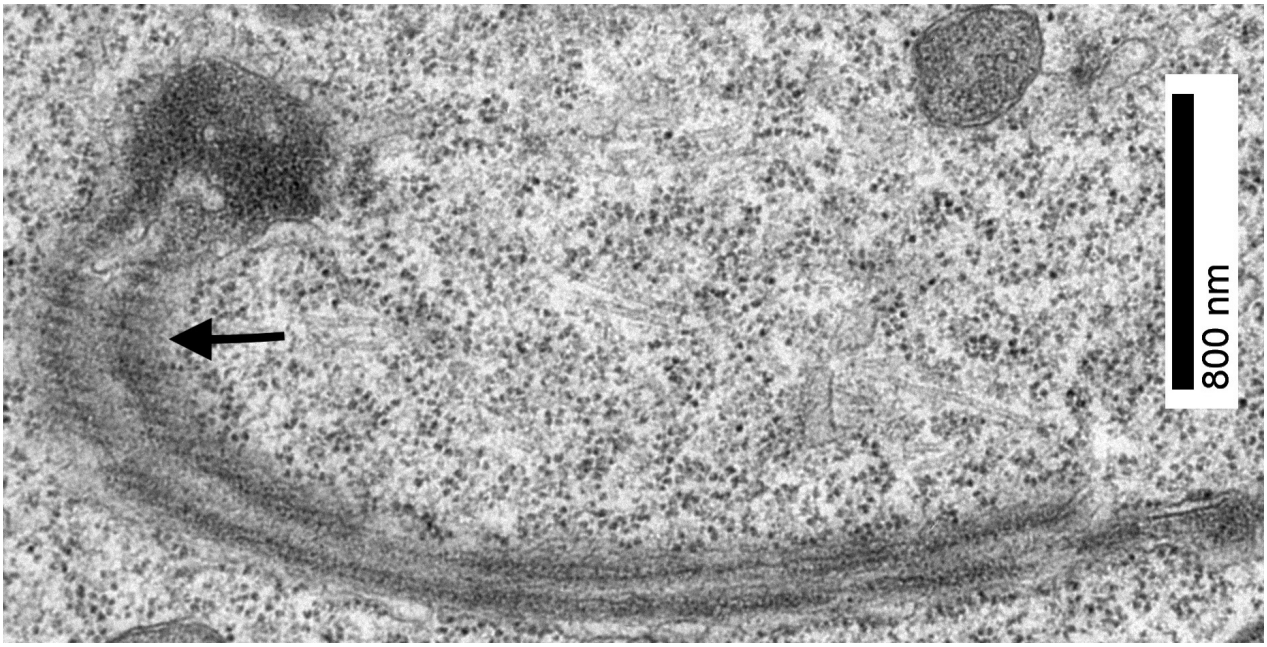


Figure 3. Two parallel rows of electron density associated with rER. The arrow points to a portion of the structure where the structures are sectioned tangentially, thus illustrating that the density is actually a rod. The dimensions of these rods corresponds to the microrods described in Fig. 1a – 1c.

A third structure, which is common in older flies, is a sphere with electron density between adjacent membranes (Figure 4a-4f). These can be elaborate with 3 or more layers of ER with density between adjacent ER membranes.

The common features of this novel organelle are, first, an outer ER with ribosomes on the cytoplasmic side and the inner membrane free of ribosomes; second, electron density adjacent to the inner ER membrane,

frequently bounded by additional ER membranes (as in Figures 2 and 3) without attached ribosomes. In Figure 2, it is clear that two separate ERs are continuous with this organelle and that an electron dense structure is found between two adjacent ER membranes. There are no ribosomes associated with this inner membrane whereas there are frequently many ribosomes on the outer membrane. The organelles seen in Figures 4 maintain the outer ribosome-studded surface and absence of ribosomes in the interior. Although we have not been able to verify this, I propose that the internal membranes are remnants of the second ER membranes. There are many examples of these spherical structures also connected to ER membranes (Figure 5a, 5b), suggestive of a mechanism by which the multilayered spheres formed.

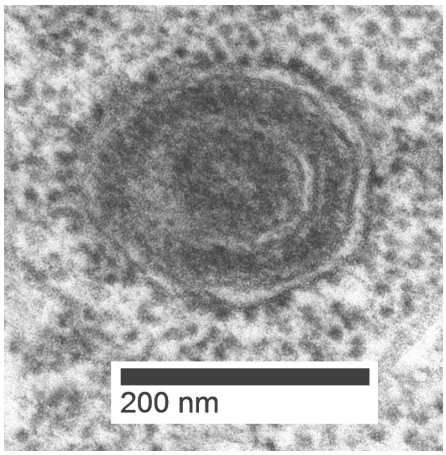


Figure 4a

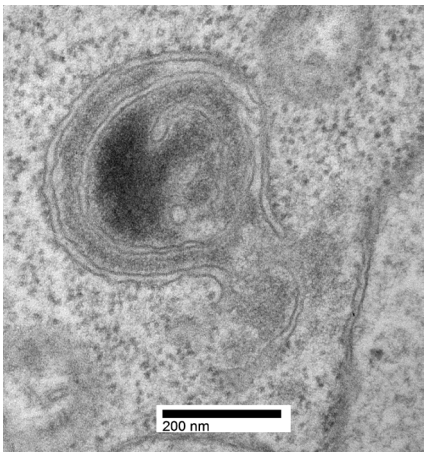


Figure 4b

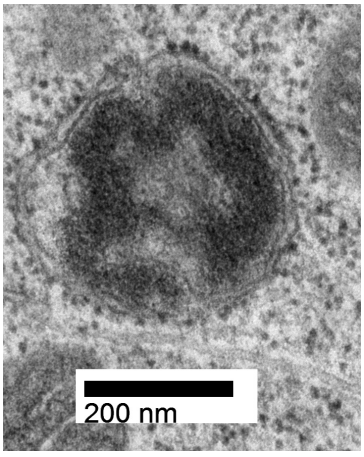


Figure 4c

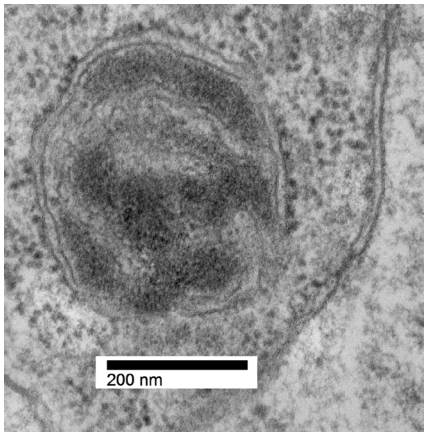


Figure 4d

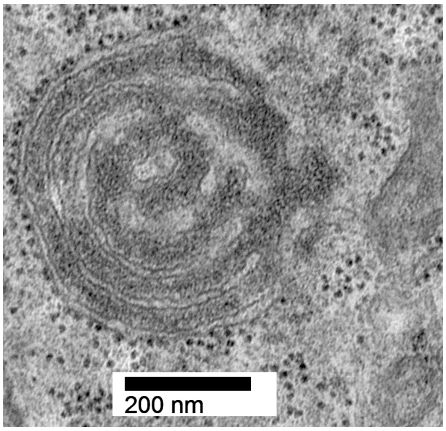


Figure 4e

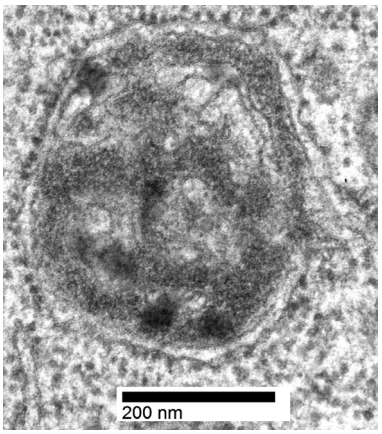
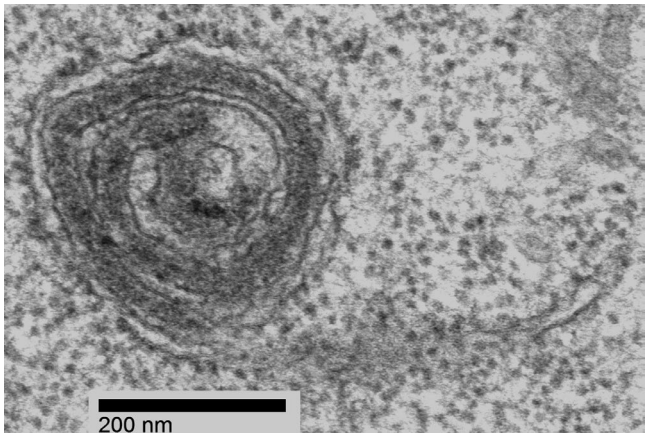


Figure 4f

Figure 4a to 4f. Common spherical structure in 3 week old flies, consisting of ER membranes surrounding a central density.

Another feature of some of these membranes is the occasional association with nuage (*cf.* Figure 2, 3). In many examples there is a clear difference in the structure and/or electron density of the nuage (Figure 6, arrow) and the density between adjacent ERs (Figure 6). Further experiments with *nanos* antibodies should clarify the relation between these two types of electron density structures.

Because the original discovery of these organelles occurred when I was studying older ovaries (2 to 3 weeks old), I also searched extensively in ovaries of one day old flies for these organelles. In 3-week-old flies I could always find structures illustrated in Figure 4 but I did not find these structures in young fly ovaries, even after scanning 50 consecutive thin sections.



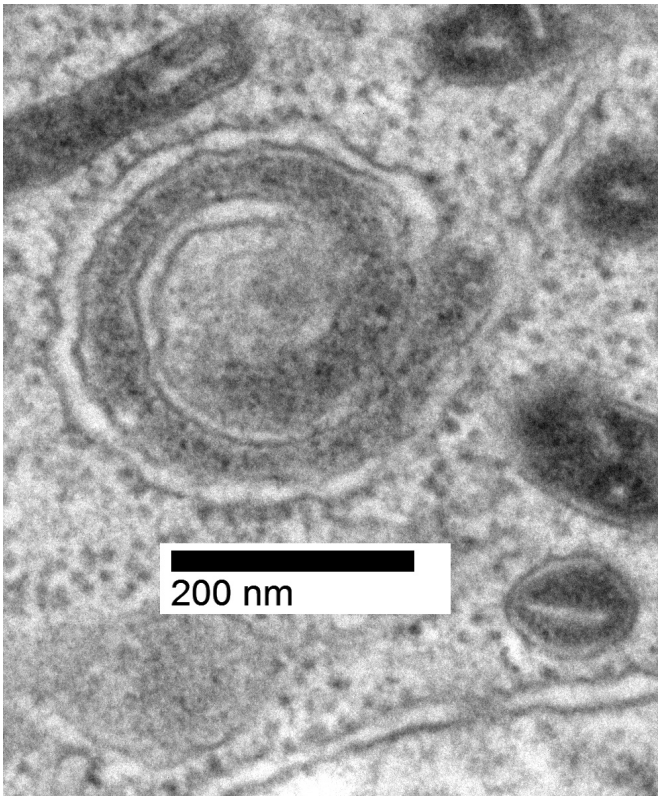


Figure 5a, above; and 5b, right. ER membrane continuous with the spherical organelle, suggesting a possible way in which the multiple layers found in Figure 4 might have formed.

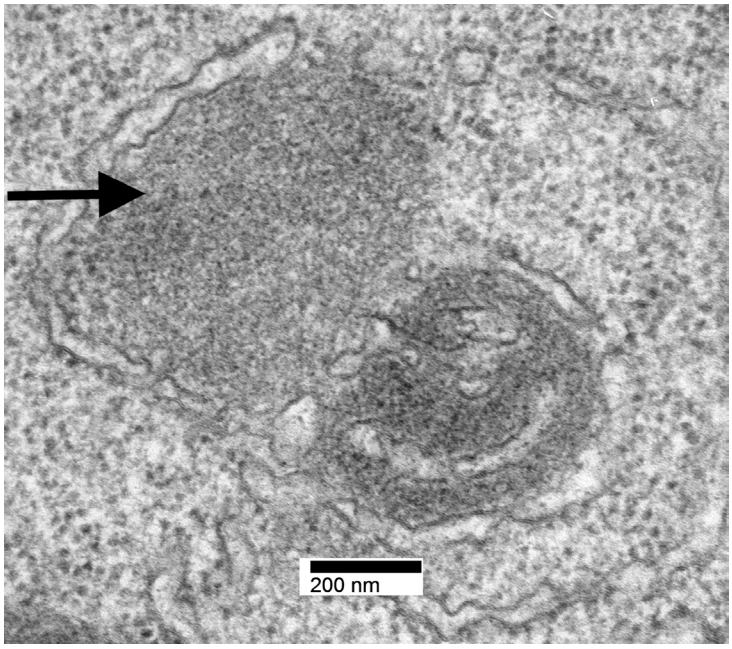


Figure 6. Nuage (arrow) closely associated with the spherical structure described in this article.

To summarize, I am describing a new structure, found in older flies, consisting of two rER organelles, back-to-back, with a thin row of electron density between the adjacent ER membranes. Because of the clear evidence seen in Figure 2 that initially the two adjacent ER organelles surround an electron-dense layer with the attached membranes without ribosomes, I propose that this is a general characteristic of this novel structure and the loss of ribosomes on the inner surface (*cf.* Figures 1, 3 and 4) may be a consequence of the close association of the two ERs.

What are the "microrods"?

When I displayed this work as a poster at a national *Drosophila* conference and at an annual meeting of the Cell Biology Society, Welcome Bender (Harvard Medical School) viewed the poster and alerted me to the amazing size similarity of the microrods to the X-ray crystallography studies of Sun Hur on the structure of double stranded RNA (dsRNA) bound by receptor proteins (Wu *et al*., 2013; Peisley *et al*., 2013). Molecular analysis of these vertebrate structures established that they are 9 nm in diameter with a central hole of 2 – 3 nm occupied by the dsRNA. A similar structure has been described for dsRNA bounded by dicer-2 protein. dicer-2 is the only homologue in *Drosophila* to the vertebrate receptors analyzed by Hur. These structures are remarkably similar to the dimensions of the "microrods" described in this paper. With advances in cryo-electron microscopy, it should be possible to determine whether the "microrod" is actually dsRNA bound to receptors like Dicer-2 protein.

Discussion

I initiated these electron microscopic studies of the germarium in order to describe the ultrastructure of the escort cells responsible for the transport of molecular signals between the germline and surrounding escort cells (Benisch *et al*., 2017). My original study used old flies (~3 weeks) from population cages maintained in T. Orr-Weaver's laboratory. Subsequently I have shown that similar structures are found in laboratory stocks that are maintained in bottles, open to the environment via a mesh instead of isolated by a tight cotton plug. The presence of the unusual structures illustrated in Figures 1, 2 and 3 were intriguing and at first, I thought they might be restricted to population cage flies which were in free contact with surrounding environment. However, I have found that I can find the same structures in a laboratory stock that has been aged for 3 weeks in a container open to the environment by a mesh. This led to the further search for other examples of these structures, which led to the observations that any stock I studied, after being aged at least 3 weeks, displayed structures seen in Figures 4-4f. This has led to the conclusion that I have identified a new structure with a potential identification of a function.

The next goal will be to verify the identity of microrods. The remarkable similarity in dimensions to that determined for vertebrate receptors bound to dsRNA is a start. Antibodies are available for dsRNA as well as for *dcr-2*, the only protein related to the mammalian receptors. Since conditions have been found for inducing the formation of microrods (*cf*., Figure 1c), these cells should be positive for dsRNA antibodies. If positive, then identifying these structures at the EM level is feasible. Similarly, a variety of antibodies are available for dsr-2 protein.

References: Benisch, Torsten U., I. Maimon, T. Dadosh, and L. Gilboa 2017, Development 144: 1937-1947; King, R.C., 1970, *Ovarian Development in* Drosophila melanogaster. Academic Press, New York; Mahowald, A.P., and J.M. Strassheim 1970, J. Cell Biol. 45: 306-320; Peisley, A., B. Wu, H. Yao, T. Walz, and S. Hur 2013, Mol. Cell 51: 573-583; Tazuke, S.I., C. Schulz, M. Fogarty, A.P. Mahowald, A. Guichet, A. Ephrussi, G.C. Wood, R. Lehmann, and M.T. Fuller 2002, Development 129: 2529-2539; Wu, Bin, Alys Peisley, Claire Richards, Hui Yao, Xiaohui Zeng, Cecilie Lin, Feixia Chu, Thomas Walz, and Sun Hur 2013, Cell 152, 276–289.

Lethal deletion associated with male recombination using the Tonga strain of *Drosophila ananassae.*

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Introduction

The spontaneous occurrence of male recombination in *Drosophila ananassae* was independently observed by Kikkawa (1937) and Moriwaki (1937), and Matsuda *et al*. (1993) provided a comprehensive overview and characterization of the genetic factors governing this phenomena. Sato *et al*. (2000) showed that the second chromosome of *e pi; bri ru* strain carries an enhancer, *En(2)-ep*, of male recombination and also found that recombination is enhanced in both sexes within a specific region between the centromeric regions of chromosome 2, *Om*(*2C*), and *Arc* in heterozygotes of the *e pi; bri ru* and *Om*(*2C*) *Arc* strains. The degree to which the recombination is increased remains undetermined. The recombination rate increased by 30- to 40-fold in males and 13- to 30-fold in females.

The wild-type Tonga strain harbors multiple enhancers on chromosomes 2L, 2R, and 3R, and their effects are presumed to be additive (Matsuda and Tobari, 1983). In the heterozygous e *se; bri ru* strain, which carries the dominant suppressor *Su(2)-bs* on over 70% of chromosomes in the natural population (Mastuda *et al*., 1993), the *Su(2)-bs* suppressor completely suppresses recombination (Tobari *et al*.,1983). Nevertheless, this suppressor has no impact on the Tonga strain. Additionally, Matsuda (1989) revealed that the Tonga strain also carries a cytoplasmic suppressor. Male recombination in *D. ananassae* arises during meiosis and is linked to chromosome breaks (Matsuda *et al*., 1983; Goni *et al*., 2006); however, the underlying mechanism remains elusive. In this paper, I employed the Tonga strain to investigate the features of recombinant chromosomes resulting from exchange events within crossing-over regions.

Materials and Methods

Strains

This study utilized three *Drosophila ananassae* strains: one wild-type strain, Tonga, and two mutant strains, *ca Om(2C)8 Arc* and *Sb/*e^D. The Tonga wild-type strain originates from the Tonga Islands and possesses several dominant enhancers and cytoplasmic repressors of male recombination (Matsuda and Tobari, 1987; Matsuda, 1989; Matsuda *et al*., 1993). The *ca Om(2C)8 Arc* strain harbors two dominant genes expressed in homozygotes, allowing for the distinction between heterozygous and homozygous phenotypes. The *Sb/* e^D, NG2 strain displays the ebony dominant body color phenotype due to the e^D gene and the dominant bristle mutant phenotype on chromosome 2L due to the *Sb* gene. The e^D mutant harbors multiple inversions on chromosome 2, and it is called In(2L+2R) NG2, with the abbreviation NG2 (Futch, 1966). Additionally, NG2 carries at least one recessive lethal mutation, and this chromosome essentially eliminates recombination throughout the entire second chromosome of ST/NG2 flies (Matsuda, 1991). Subsequently, I utilized this strain as the balancer chromosome for the second chromosome. The genetic code adheres to the description provided by Tobari (1993).

Mating scheme

Each F_1 male or female derived from reciprocal crosses between the Tonga and *Om(2C) Arc* strains was subsequently crossed with the e^D*/Sb* balancer strain. The recombination frequencies of *Om(2C)* and *Arc* in both sexes were assessed employing the identical procedure outlined by Matsuda and Tobari (1983). The genetic distance between *Om(2C)* and *Arc*, as determined by a standard genetic map, is approximately 0.4% (Tobari, 1993). Additionally, the cytological locations of *Om(2C)* and *Arc* are on the right side of bands 44C

and 45C, respectively (Matsubayashi *et al*., 1993; Sato *et al*., 2005). The balancer chromosome served as a tool for isolating the recombinant chromosome. Male recombinant flies of the genotypes *Arc*/e^D,* NG2 or *Om(2C)*/e^D*, NG2 were individually crossed with *Sb/e^D*, NG2 females to produce *Arc*/e^D* NG2 or *Om(2C)*/e^D* NG2 flies possessing the same *Arc** or *Om(2C)** chromosomes. Upon inbreeding the daughters and sons of *Arc*/e^D*, NG2 or *Om(2C)*/e^D*, NG2 from the aforementioned crosses, the presence or absence of homozygous *Arc/Arc* or *Om(2C)/Om(2C)* offspring indicated the presence or absence of recessive lethality in their recombinant chromosomes. Each identified recombinant chromosome was preserved in a heterozygous state with *e^D*, NG2 chromosomes, and allelic deletions were detected through complementation testing. A recombinant fly was classified as semi-lethal if less than 5% of the offspring resulting from inbreeding exhibited homozygosity for *Arc/Arc* or *Om(2C)/Om(2C)* flies. Experiments were conducted under controlled conditions at 25°C using standard cornmeal, yeast, glucose, and agar media.

Results and Discussion

Sex-specific recombination frequency

The average recombination frequencies between *Om (2C)* and *Arc* in both F1 males and females are shown in Table 1. The frequency in F1 females between Tonga female and marker male is 0.6%, which is very close to the standard genetic map of 0.4%. On the other hand, three other hybrids of different genotypes produced high frequency recombination. The magnitude of the increased frequency is about 60-fold, 7-fold and 15-fold in F1 males and females between *Om (2C) Arc* female and Tonga male and the reciprocal F1 males. There is no clustering of recombinant progeny among individual crosses (data not shown). Although the range of recombination frequencies is variable, the lowest frequency in the above hybrids at least shows the higher than the standard map distance.

Table 1. Mean recombination frequency in F_1 males and females from reciprocal crosses between *Om(2C) Arc* and ++ (Tonga) strains.

Crosses (female x male)	Number of crosses observed	Number of progeny observed	% recombination between Om(2C) and Arc (Ranger)
Om(2C) Arc x Tonga			
F_1 female	10	1366	3.9 (2.3~ 9.9)
F_1 male	13	2636	17.7 (15.1~23.7)
Tonga x *Om(2C) Arc*			
F_1 female	6	1599	0.6 (0.0~ 1.2)
F_1 male	14	3739	8.6 (3.9~12.0)

Prevalence of recessive lethality in recombinant chromosomes

Table 2 presents the distribution of chromosomes carrying recessive lethal and semi-lethal alleles within the recombinant chromosome, [*Om(2C)*] and [*Arc*], derived from F_1 hybrids generated by crossing Tonga and *ca Om(2C) Arc* strains. The highest frequency (27.1%) of the recessive lethal-carrying [*Arc*] recombinant chromosome was observed among the progeny of F_1 males generated from crosses between *Om(2C) Arc* females and Tonga males. A high prevalence of recombinants was observed in both F_1 males and females derived from Tonga male parents and F_1 males obtained from Tonga female parents. No statistically significant correlation was detected between recombination and lethal frequencies. Notably, two of 54 non-recombinant [*Om(2C) Arc*] chromosomes from *Om(2C) Arc*/++ F_1 males harbor recessive lethal alleles. No rearrangements were detected in polytene chromosomes of all lethal chromosomes isolated in the experiment.

Table 2. Distribution and prevalence of recombinant chromosomes carrying recessive lethal and semi-recessive lethal alleles originating from both F_1 males and females in the *Om(2C) Arc* and Tonga strains.

Genotype F1 progeny	Phenotype of recombinant carrying	No. of recombinant carrying recessive lethal tested	No. of carrying recessive lethal	% chromosome (Female/Male) chromosome recessive
Om(2C) Arc/++ (males)				
	[Arc]	48	13	27.1
	{Om(2C)]	72	6	8.3
	Total	120	19	15.8
Om(2C Arc/++ (females)				
	[Arc]	8	0	0.0
	{Om(2C)]	11	1	9.1
	Total	19	1	5.3
++/Om 2C) Arc (males)				
	[Arc]	23	2	8.7
	{Om(2C)]	38	0	0.0
	Total	61	2	3.3

Genetic complementation test of recessive lethal mutations

A total of 24 lethal and semi-lethal chromosomes were independently isolated from the recombination experiments and employed for complementation testing between lethal genes. Chromosomes harboring lethal alleles, designated as [Om] or [Arc] recombinant chromosomes, were obtained from F1 males produced by crosses between Om(2C) Arc females and Tonga males. In contrast, rOm or rArc referred to as [rOm] or [rArc] recombinant chromosomes, respectively, were obtained from F_1 males resulting from crosses between Tonga females and Om(2C) Arc males. Analysis of 21 lethal chromosomes revealed the existence of 6 complementation groups (Table 3).

Table 3. Lethal complementation groups determined for 24 lethal chromosomes.

Group 1	Om1, Om2, Om3, Om4, Om5, Arc3, Arc4, Arc5, Arc6, Arc10, Arc11, Arc12, Arc13, OA1, fOm1
Group 2	Om6, Arc1
Group 3	Arc2
Group 4	Arc7
Group 5	Arc8
Group 6	Arc9
Group 7	OA2
Group 6	rArc1, rArc2

Although 15 out of 24 lethal chromosomes are classified within a single group, they are not fully allelic, except for Arc5 and Arc10, and Arc6 and Arc11. Within this group, complementation is observed between some pairs, while others do not complement, leading to the formation of a "partial allelic group." Each of the remaining five complementation groups consists of a single lethal chromosome.

As shown in Table 4, the relationship between lethal regions on chromosomes based on allelic testing was elucidated employing the 15 lethal chromosomes of complementation group 1. Despite the complexity of

this relationship, the occurrence of lethal crossover events seems to be linked to chromosomal deletions, with varying lengths of these deletions observed.

Table 4. Complementation test results among 15 chromosomes of Group 1 carrying lethal alleles

	Arc3	Arc12	Om5	fOm1	Om2	Om4	Om3	Om1	OA1	Arc5 Arc10	Arc6 Arc11	Arc4	Arc13
Arc3	x	△											
Arc12		x	x									△	
Om5			x	△	x	△							
fOm1		△	×	×	△	x	x						
Om2					x		△	△					
Om4						x	x	△	x	△			
Om3							x	△	x	X	X	x	
Om1								x	x	X	X	x	
OA1									x	△	X	x	
Arc5 Arc10										X	X	x	
Arc6 Arc11											X	x	
Arc4												x	x
Arc13													X

x: lethal △ : semi-lethal

Acknowledgments: In memory of the late Dr. Yoshiko N. Tobari, who passed away on February 12, 2022, I would like to express my deepest gratitude for her invaluable contributions and enduring support throughout this research endeavor.

References: Futch, D., 1966, Univ. Texas Publ. 6615: 79-120; Goni, B., M. Matsuda, and Y.N. Tobari 2006, Genome 49: 1374-1383; Hinton, C.W., and J.E. Downs 1975, Journal of Heredity 66: 353-361; Kikkawa, H., 1938, Genetica 20: 458-516; Matsuda, M., 1989, Japanese Journal of Genetics 64: 99-110; Matsuda, M., 1991, Japanese Journal of Genetics 66:701-707; Matsuda, M., 1993, *In*: Drosophila ananassae-*Genetical and Biological Aspects*. (Tobari, Y.N., ed.), Japan Scientific Societies Press and Karger, Tokyo pp.139-138; Matsuda, M., H. Sato, and Y.N. Tobari 1993, *In*: Drosophila ananassae-*Genetical and Biological Aspects*. (Tobari, Y.N., ed.), Japan Scientific Societies Press and Karger, Tokyo pp.53-72; Matsuda, M., Y.N. Tobari, and H.T. Imai 1983, Chromosoma 88: 286-292; Matsuda, M., and Y.N. Tobari 1983, Japanese Journal of Genetics 58: 181-192; Matsuda, M., and Y.N. Tobari 1987, Japanese Journal of Genetics 62: 217-224; Moriwaki, D., 1938, Japanese Journal of Genetics 14: 283-284; Moriwaki, D., and Y.N. Tobari 1993, Drosophila ananassae-*Genetical and Biological Aspects* (Tobari, Y.N., ed.), Japan Scientific Societies Press and Karger, Tokyo, pp 49-51; Sato, H., B. Goni, M. Matsuda, and Y.N. Tobari 2000. Genes and Genetic Systems 75: 41-47; Tobari, Y.N., 1993. Drosophila ananassae-*Genetical and Biological Aspects*. (Tobari, Y.N., ed.), Japan Scientific Societies Press and Karger, Tokyo, pp. 49-51.

Teaching note: How does a fly walk?

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Walking is one of the activities that most characterize animals, including humans. In nature and in the laboratory, the walking of adult *Drosophila* is related to: i) identification of conspecific and heterospecific flies, ii) searching for food sources, iii) escaping from predators and more. The male's courtship of the female before copulation also includes walking. Adult female *Drosophila* walk when they select places to lay the eggs. Therefore, walking in animal species is a behavior involved in selection and occupation of habitats.

In this practical, how male and female *Drosophila* walk in a laboratory environment will be recorded. The collected data will then be processed and the way adults of each sex move will be compared.

Biological Material

Groups of 2 - 3 students will be provided in separate vials with male and female *D. melanogaster*. Walking of individuals of the same species and sex, but of different ages, can also be recorded. The method also allows comparing the movement patterns of individuals of different *Drosophila* species.

Procedure to collect data

1.- Aspirate a male (female) into a 2.5×10.0 cm vial that has not contained adults of any species.

2.- After 2 minutes of acclimatization to the new environment, record in sequence the different behavioral categories exhibited by individuals tested.

Our experience is that recording continuously each tested fly for 2 min is enough.

3.- Record the data with the naked eye. Use check sheets, one per individual, as described in Martin and Bateson (2007, pages 64 – 67).

A check sheet is a grid with columns denoting different behaviors and rows denoting successive sample intervals. In this way a flow chart can be generated. That is, each check sheet is a picture of the different separate behaviors in sequential order that a female (male) did.

4.- Before starting the records, it is essential that students all together observe what the flies do in the vials. Between 4 – 5 min is enough. In this way the group agrees on what behaviors to record.

5.- In our experience, recording a sample of n = 8 females (males) provides satisfactory information on how a male (female) walks.

6.- Time. The practical takes between 2.30 – 3 h.

7.- Once finished with the activity, students must write a report: Title, Introduction, Materials and Methods, Results, Discussion, References.

Some major conclusions – Listen from the students

1.- The behaviors exhibited by animals, including humans, are indicators of how the nervous system is functioning.

2.- The male and female *Drosophila* walk differently, which suggests that the respective nervous systems function differently.

3.- For over 18 years we have listened to undergraduate and graduate students:

3.1.- "We are surprised that an activity seemingly as simple as going from one place to another, involves an exquisite neuro-motor coordination by the nervous system of flies like *Drosophila*."

3.2.- "We could use neurological mutations to know whether they modify walking in adult *D. melanogaster.*"

References: Martin, P., and P. Bateson 2007, *Measuring Behaviour*. Cambridge University Press. Cambridge, UK.

Geographical distribution of *Zygothrica* species (Diptera: Drosophilidae) in Brazil, with comments on taxonomic aspects.

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Abstract

The Neotropical species of *Zygothrica* (Diptera: Drosophilidae) lodged in the Entomological Collection of the Instituto Oswaldo Cruz (CEIOC), Rio de Janeiro, RJ, Brazil, is revised. Thus, we identify specimens collected in several localities from Brazil, present figures with the external morphology and terminalia and update the current known distribution of several species. Eighteen species identified, besides the male terminalia of *Zygothrica neoaldrichi* Burla 1956 are there presented here. In addition, new occurrences for 15 species were recorded. Key-words: Neotropical biodiversity, mycophagy, Drosophilinae, new record, taxonomy.

Introduction

Zygothrica Wiedemann (1830) is a genus is predominantly Neotropical and has around 130 valid species (Bächli, 2020), out of which 54 occur in Brazil (Grimaldi, 1990; Gottschalk *et al*., 2008; Robe *et al*., 2014; Tidon *et al*., 2017). The most substantial contribution was the study proposed by Grimaldi (1987), where there were descriptions of 49 new species, but since 1987s no new species descriptions of this genus have been reported. Here, we identified the *Zygothrica* species lodged in the Entomological Collection of the Instituto Oswaldo Cruz (CEIOC), Rio de Janeiro, RJ, Brazil. Therefore, we present the morphology of 18 known species of *Zygothrica*, besides the male terminalia of *Z. neoaldrichi* Burla 1956. Furthermore, we present new distribution records for 15 species.

Materials and Methods

The identification of individuals was performed based on external morphology and in the analysis of male or female terminalia, which were prepared following the protocol adapted from (Bächli *et al*., 2004; Mendes and Gottschalk 2019). The terminalia was disarticulated in glycerin and mounted on temporary microscope slides with glycerin jelly (aqueous solution of 2% gelatin and 50% glycerin) (Grimaldi, 1987) for illustration and photographic records. After, the microscope slides were dismounted and the genital sclerites were placed in microtubes with glycerin and attached to the individuals.

The individuals selected for each species were there photographed with a Zeiss Discovery V.20 photomicroscope, in lateral, dorsal and frontal views. The wing was also photographed in detail. The images were stacked by the software AxioVision 4.9.1.

The morphological nomenclature followed Cumming and Wood (2017). Measurements of body structures and color descriptions followed Grimaldi (1987) and Vilela and Bächli (2000). Complementary information added to the original labels was obtained from the database of the Instituto Brasileiro de Geografia e Estatística. The complementary information of labels is presented between brackets in the examined material. Species occurrence information was obtained from TaxoDros v.1.04 (2020/02) (Bächli, 2020).

The species identified were morphologically compared with known *Zygothrica* species, which are mainly type series deposited in collections, from photographs. Finally, all specimens studied are deposited in the Entomological Collection of the Instituto Oswaldo Cruz (CEIOC), Rio de Janeiro, RJ, Brazil.

Results

The species group arrangement followed Grimaldi (1987) and Burla (1956).

Zygothrica Wiedemann, 1830

Achias (*Zygothrica*) Wiedemann, 1830: 16. Species type: *Achias* (*Zygothrica*) *dispar* Wiedemann, 1830; by the original designation.
Drosophilura Hendel, 1913: 389. Species type: *Drosophilura caudata* Hendel, 1913; by the original designation.
Zygothrica Sturtevant, 1920: 156.
Zygothrica (*Tanyglossa*) Duda, 1925: 189. Species type: *Zygothrica* (*Tanyglossa*) *tenuirostris* Duda, 1925. Junior homonym of *Tanyglossa* Meigen, 1803: 267 (Diptera: Tabanidae).
Zygothrica Burla, 1956: 215.
Zygothrica Grimaldi, 1987: 147.

Diagnosis. See Grimaldi (1987).

atriangula group

Zygothrica atriangula Duda *sensu* Burla (Figure 1)
Zygothrica atriangula: Burla (1956).
Zygothrica "atriangula" Duda *sensu* Burla, 1956: Grimaldi (1987).

Material examined: 02 ♀ labeled as "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 01 ♀ labeled as "[Brazil, Rio de Janeiro], Paineiras, Corcovado; Janeiro de 1968; Col: M. Alvarenga". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Peru and Brazil (States of Rio de Janeiro and Pará).
Comments: According to Grimaldi (1987), *Z. atriangula sensu* Duda (1927) and *sensu* Burla (1956) are not the same species and, to date, the identity of these species has not been clarified. Here we adopt *Z. atriangula sensu* Burla (1956).

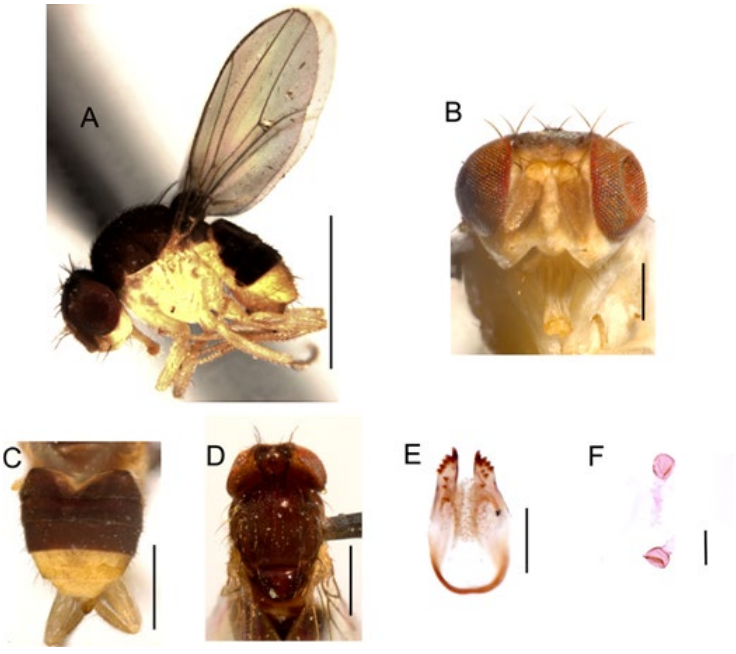


Figure 1. *Zygothrica "atriangula"* sense Burla ♀. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0.1mm).

Zygothrica virgatinigra Burla (Figures 2-3)
Zygothrica virgatinigra Burla, 1956: 252.

Type locality: Rio de Janeiro, RJ, Brazil.
Material examined: 03 ♂, 02 ♀ labeled as "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Brazil (States of Rio de Janeiro, São Paulo and Pará [new record]).

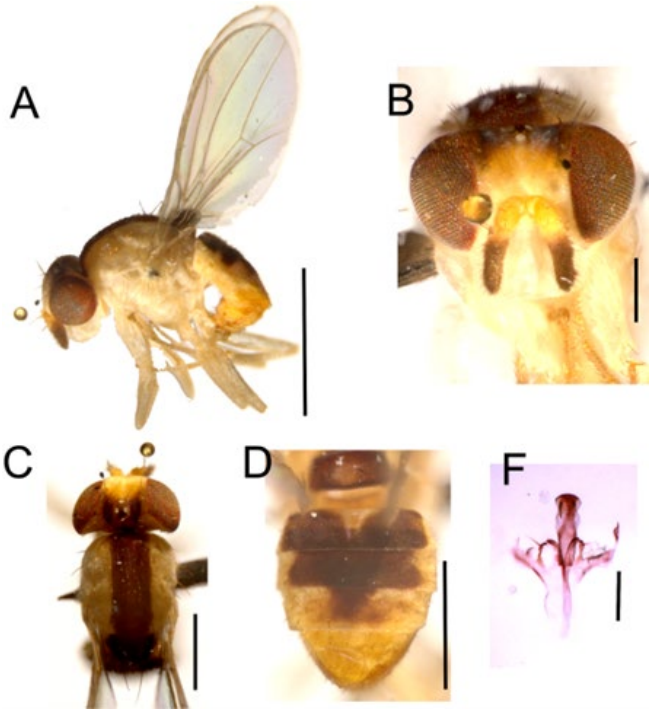


Figure 2. *Zygothrica virgatinigra* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: phallus, phallapodeme, hypandrium, pregonites and postgonites, ventral view (scale bars: 0.1mm).

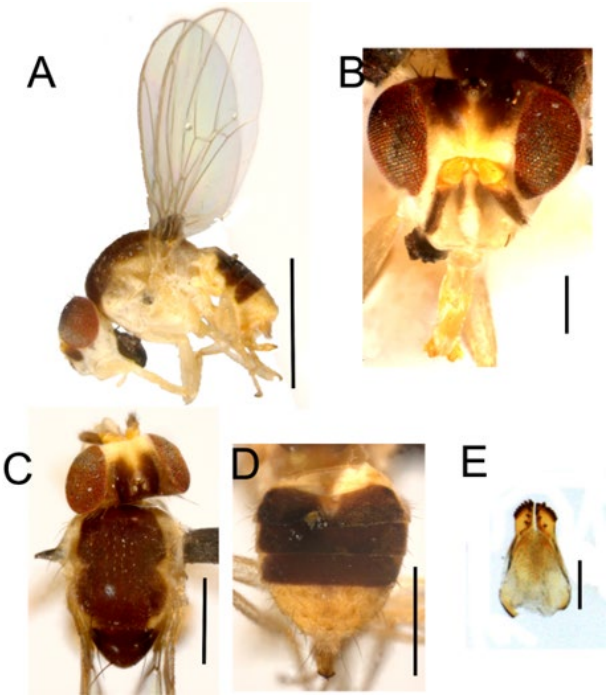


Figure 3. *Zygothrica virgatinigra* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view (scale bars: 0.1mm).

bilineata group

Zygothrica bilineata (Williston) (Figures 4-5)
Drosophila bilineata Williston, 1896: 409.
Zygothrica gemma Burla, 1956: 249.

Type locality: Saint Vicente, West Indians.
Material examined: 02 ♂, 01 ♀ labeled as "[Brazil], Go[i]as, [Goiânia]; 21.XII.[1]936; Col: Borgmeier et S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Colombia, Brazil (States of Goiás [new record], Mato Grosso, Pará, Paraná, Rio de Janeiro, Rio Grande do Sul, Rondônia, Santa Catarina and São Paulo) and Peru.

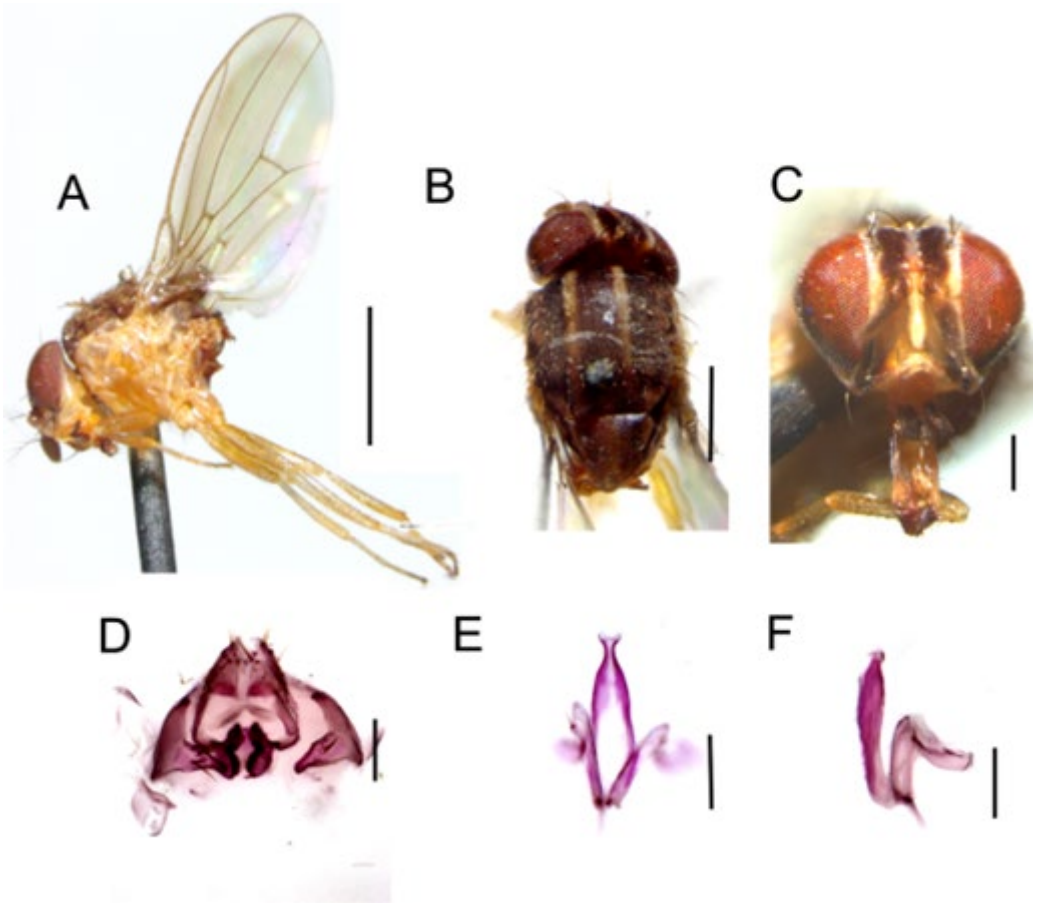


Figure 4. *Zygothrica bilineata* ♂. A: lateral view; B: thorax, dorsal view; C: head, frontal view (scale bars: 0.5mm; B: 0.2mm). D: epandrium, cerci, surstyli and Subepandrial Sclerite in posterior view; E: phallus, phallapodeme, and pregonites, ventral view; F: phallus and phallapodeme, lateral view (scale bars: 0.1mm).

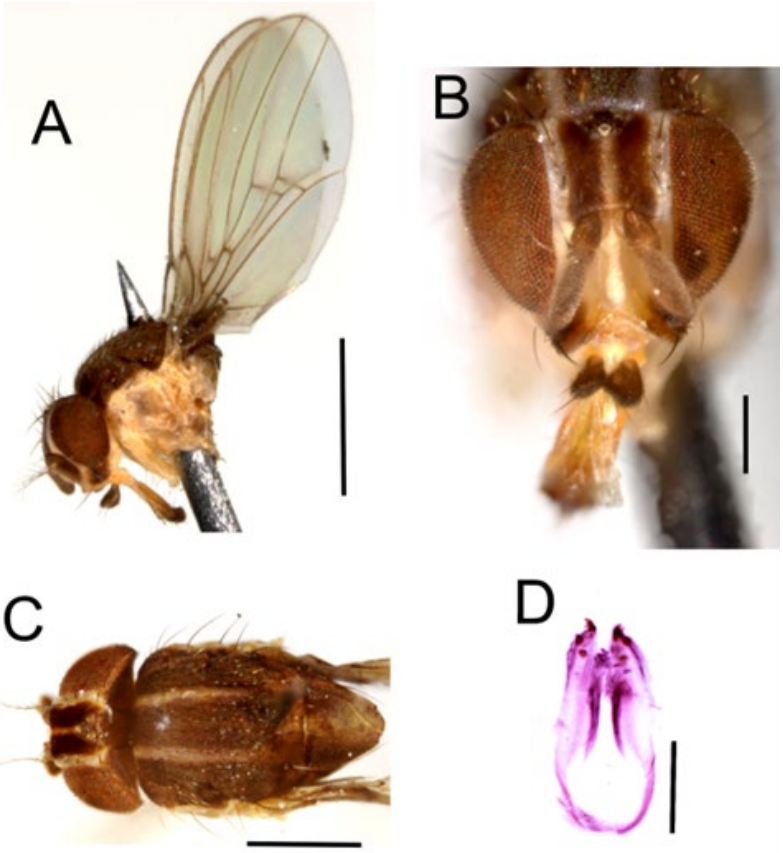


Figure 5. *Zygothrica bilineata* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). D: oviscapt (scale bar: 0.1mm).

dispar group
dispar subgroup

Zygothrica dispar (Wiedemann) (Figures 6-7)
Achias dispar Wiedemann, 1830: 556.

Type locality: The original description mentioned "Brasilia", probably a reference to Brazil, once Brasília (federal district of Brazil) was founded in 1960.
Material examined: 03 ♂ labeled as "[Brazil], Go[i]as, [Goiânia]; 02.I.[1]936; Col: Borgmeier et S. Lopes". 03 ♂, 07 ♀ labeled [Brazil], [Rio de Janeiro], Paineiras, Corcovado; Janeiro de 1958; Col: M. Alvarenga". 01 ♀ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 01 ♀ labeled "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L[auro]. Trav[assos]. F[ilho]; VII.[1]936; Col: L. Trav.". 02 ♀ labeled "Brasil, São Paulo, Ilha Seca, Com. I[nstituto] O[swaldo] C[ruz]; 18/26.II.1940". 01 ♀ labeled "[Brazil], Goiás, Anápolis; 01.VI.[19]36". The abdominal sclerites and the terminalia were stored in microvias with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Brazil (States of Espírito Santo, Goiás (Anápolis) [new record], Pará, Piauí, Rio de Janeiro, Santa Catarina and São Paulo), Paraguay, Peru and Venezuela.
Comments: The females of this species is very similar to *Z. prodispar* females, which are mainly distinguished by the spermathecal capsule morphology and by the oviscapt plates dorsoventrally flattened. Females of *Z. dispar* have the apical margin of the oviscapt plate oblique, with 9-10 prensisetae decreasing in size to apex (Fig. 7E), while *Z. prodispar* have oviscapt plates dorsoventrally flattened with terminal row of ovisensilla and 7-8 prensisetae (Fig. 10E) (Grimaldi, 1987).

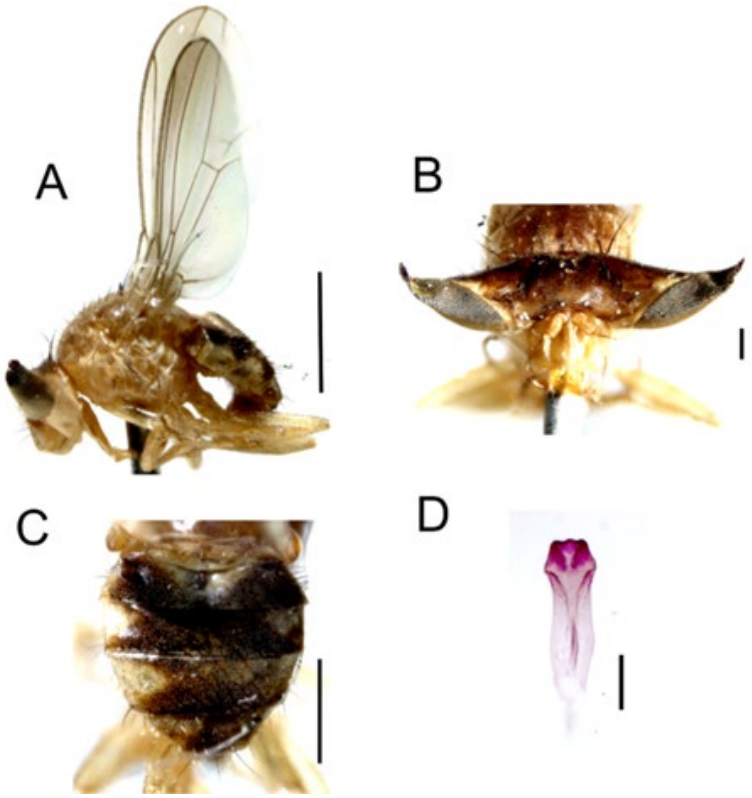


Figure 6. *Zygothrica dispar* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). D: phallus and phallapodeme, ventral view (scale bars: 0.1mm).

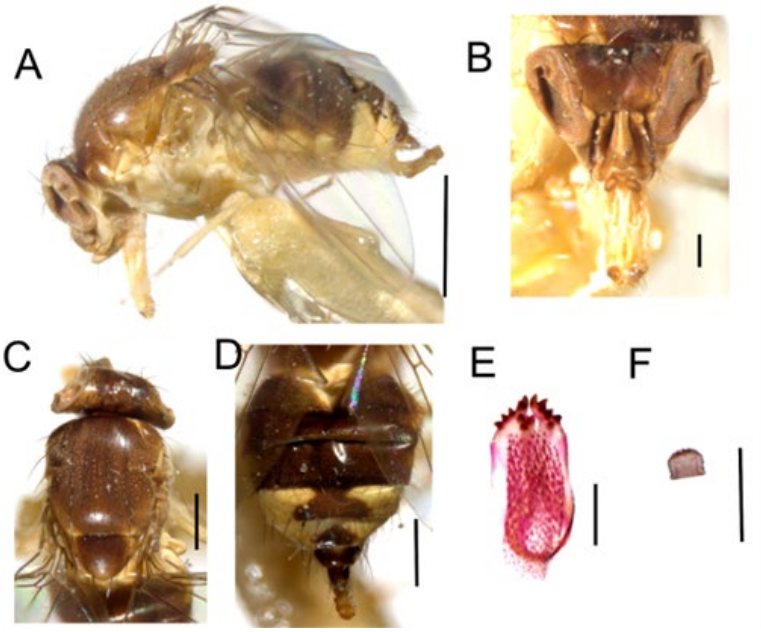


Figure 7. *Zygothrica dispar* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0.1mm).

Zygothrica exuberans Wheeler (Figure 8)
Zygothrica exuberans Wheeler, 1968: 438.

Type locality: Ecuador, Pichelingue.

Material examined: 01 ♀ labeled as "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L. Trav. F.; VII.[1]935; Col: L. Trav.". 01 ♀ labeled "[Brazil], Go[i]as, [Goiânia]; 02.I.[1]936; Col: Borgmeier et S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Brazil (State of Rio de Janeiro (Angra dos Reis) and Goiás (Goiânia) [new record]), Ecuador and Peru.
Comments: The female of this species is similar to *Z. dispar*, which are mainly distinguished by the spermathecal capsule morphology, that is square-shaped in lateral view and the cupule is bare in *Z. exuberans* (Fig. 8B), but rounded in lateral view and with scales in the external surface in *Z. dispar* (Fig. 7F) (Grimaldi, 1987).

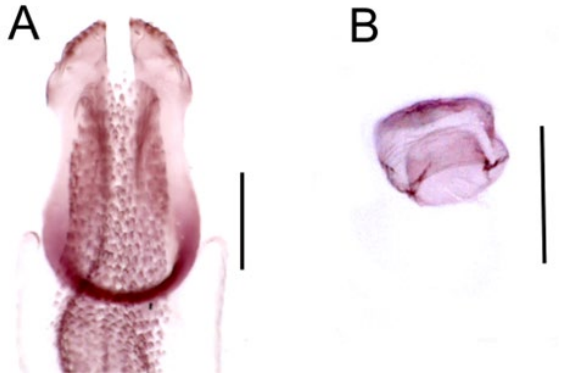


Figure 8. *Zygothrica exuberans* ♀. A: oviscapt; B: spermatheca (scale bars: 0.1mm).

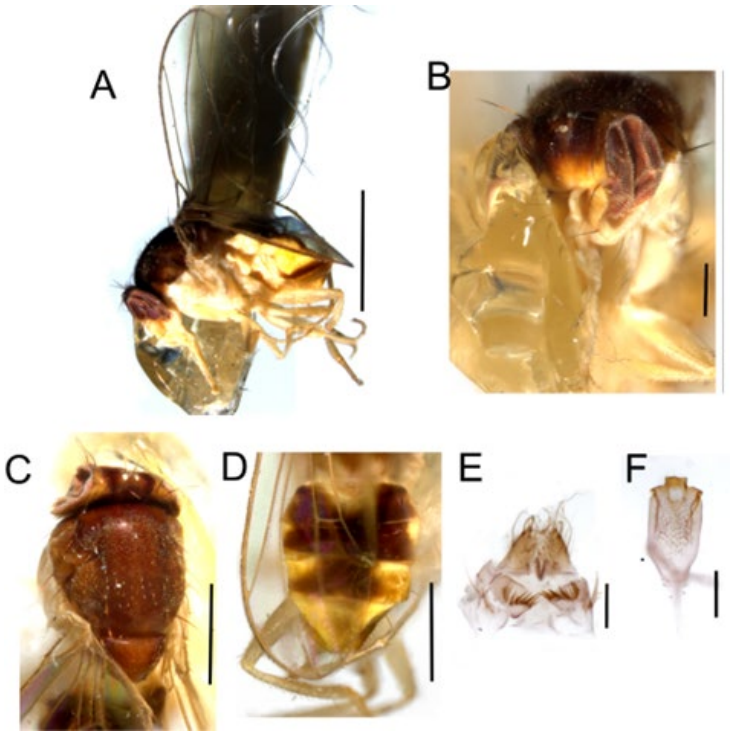


Figure 9. *Zygothrica prodispar* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: epandrium, cerci, and surstyli, posterior view; F: phallus and phallapodeme in ventral view (scale bars: 0.1mm).

Zygothrica prodispar Duda (Figures 9-10)
Zygothrica prodispar Duda, 1925:189.

Type locality: Pinipini, Peru.

Material examined: 02 ♂, 09 ♀ labeled as "[Brazil], [Rio de Janeiro], Paineiras, Corcovado; Janeiro de 1958; Col: M. Alvarenga". 02 ♀ labeled "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L. Trav. F.; VII.[1]936; Col: L. Trav.". 01 ♀ labeled "[Brasil], Minas [Gerais], Lassance; 20/31.I.[19]39; Col: Martins, Lopes e Mangabeira". 01 ♀ labeled "[Brazil], São Paulo, Cantareira, Horto Florestal; L. Trav. F; 3.[1]935; Col: L. Trav.". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Brazil (States of Bahia, Mato Grosso, Minas Gerais [new record], Pará, Piauí, Rio de Janeiro, Santa Catarina and São Paulo), Colombia, Ecuador, Guyana, Peru and Republic of Suriname.

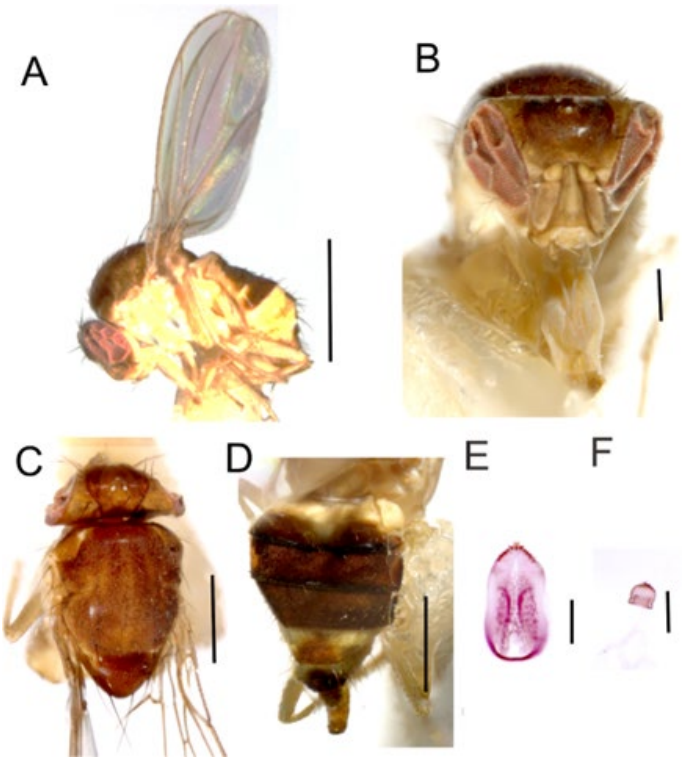


Figure 10. *Zygothrica prodispar* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0.1mm).

aldrichi subgroup

Zygothrica aldrichii Sturtevant (Figure 11)
Zygothrica aldrichii Sturtevant, 1920: 157.

Type locality: Panamá.
Material examined: 01 ♀ labeled as "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 01 ♀ labeled "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L. Trav. F; VII.[1]936; Col: L. Trav.". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographic distribution: Brazil (Pará and Rio de Janeiro States [new record]), Panama and Peru.

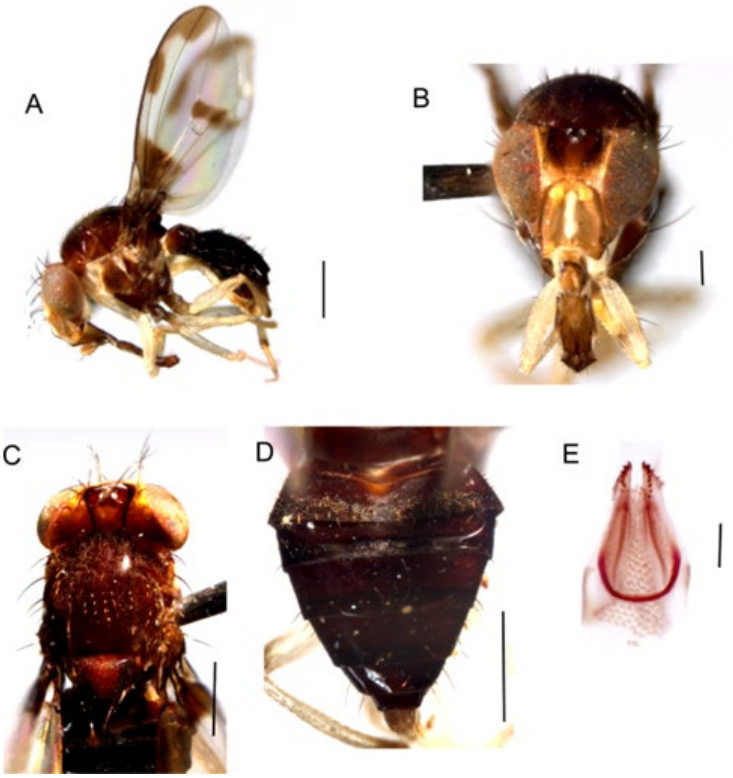


Figure 11. *Zygothrica aldrichii* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view (scale bar: 0.1mm).

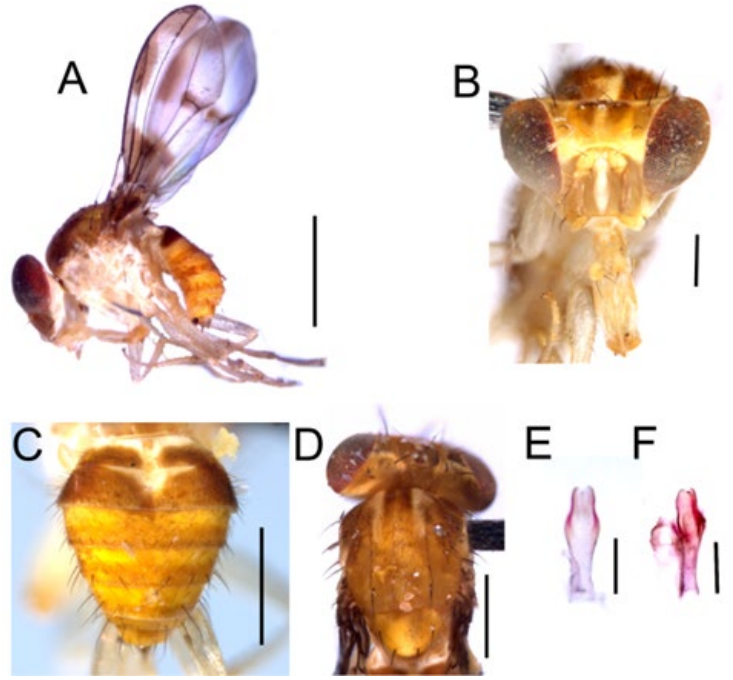


Figure 12. *Zygothrica microeristes* ♂. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). E-F: phallus, ventral and lateral view (scale bars: 0.1mm).

Zygothrica microeristes Grimaldi (Figure 12)
Zygothrica microeristes Grimaldi, 1987:218

Type locality: Belém, Pará, Brazil.
Material examined: 01 ♂ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 01 ♀ labeled "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L. Trav. F; VII.[1]936; Col: L. Trav.". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Brazil (States of Amazonas, Espírito Santo, Pará, Mato Grosso, Rondônia e Rio de Janeiro [new record]), Ecuador, Guyana, Peru and Republic of Suriname.

Zygothrica neoaldrichi Burla (Figures 13-15)
Zygothrica neoaldrichi Burla, 1956: 222.

Material examined: 01 ♂ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 02 ♀ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and were attached to the specimens.
Geographical distribution: Brazil (RJ, SP and PA (Utinga, Belém do Pará) [new record]).

Diagnosis (based on the male, to female diagnosis see Burla (1956)). Body color mainly yellow; thorax yellow; 8 parallel lines of acrostichal setulae; basal scutellar setae convergent; pleura yellow, lighter than mesonotum; abdomen yellow, with black band in tergites II-IV without reaching the lateral margin. Aristae with 4 dorsal branches, 1 ventral and 3 internal branches, plus terminal fork; flagellomere yellow, length approximately 2,5x the length of the pedicel; facial carina light yellow, prominent. Legs yellow. Wings hyaline, clouded in some regions, with R_{4+5} slightly curved in direction to M; apical spot slightly brown, restricted in the apex of R_{4+5}; spot on the last ¼ portion of R_{2+3}; r-m and dm-cu infuscate.

Description. ♂
Head. Yellow; head width = 0.93 mm. Eyes bare, dark red. Flagellomere yellow, length about 2.5x the length of the pedicel, and about 2x the width. Aristae with 4 dorsal branches, 1 ventral and 3 internal branches, plus the terminal fork. Orbital plates yellow. Orbital bristles dark brown. Distance between or1 e or2 = 0.06 mm, between or1 and or3 = 0.13 mm and between or2 and or3 = 0.07 mm. Frons light brown. Ocellar triangle dark brown, occupying 1/3 of the length of the frons; length of the frons about 1.5x the anterior width; posterior width of the frons about 2/3 of the anterior width. Light brown median frontal vitta, reaching the ptilinal suture; postocellar bristles convergent. Face and gena yellow; palps yellow, slightly darker than proboscis.
Thorax. Light brown, without distinct pattern; 8 parallel lines of acrostichal setulae; scutellum light brown; basal scutelar setae convergent; pleurae yellow, lighter than mesonotum; legs yellow. Thorax length = 1.05 mm. Thorax width = 0.77 mm.
Wings. Hyaline, absent *lappet*; wing with R_{4+5} slightly curved, brown spot bordering the sack, extending horizontally until shortly after R-M and vertically to M, elongated spot in the last portion of R_{2+3}, brown spot in the apical region of vein R_{4+5} and brown spot in dM-Cu; dumbbells yellow. Indices: C = 1.70; ac = 3.30; hb = 0.63; 4c = 0.88; 4v = 1.14; 5x = 1.1; M = 0.30; prox. x = 0.27. Length = 1.95 mm.
Abdomen. Yellow, with dark bands on tergites II-IV without reaching the lateral margin.
Terminalia. Unfortunately, the epandrium was lost in the process of preparation. Postgonites (gonopods *sensu* Grimaldi, 1987) square-shape, fused to the hypandrium; with one bristle. Pregonites (paraphysis *sensu* Grimaldi, 1987) attached to postgonites. Hypandrium V-shaped, without apparent growth lines. Phallus (basiphallus+distiphallus *sensu* Grimaldi, 1987) tubular, with basiphallus narrower than distiphallus, with an indent in apex; distiphallal scales present; length of the phallus about 5x the width of distiphallus; basiphallus fused to the phallapodeme (aedeagal apodeme *sensu* Grimaldi, 1987). Phallapodeme shorter than phallus.

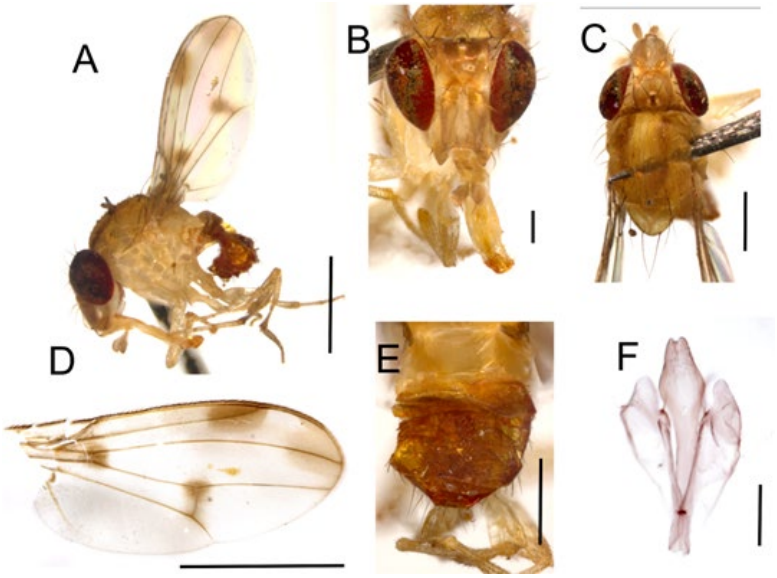


Figure 13. *Zygothrica neoaldrichi* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: wing; E: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). F: phallus, phallapodeme, hypandrium, pregonites and postgonites, ventral view (scale bar: 0.1mm).

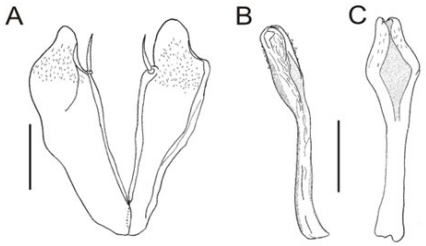


Figure 14. *Zygothrica neoaldrichi* ♂. Male terminalia. A: hypandrium, pregonites, and postgonites in ventral view; B: phallus and phallapodeme, lateral view; C: phallus and phallapodeme, dorsal view (scale bars: 0.1mm).

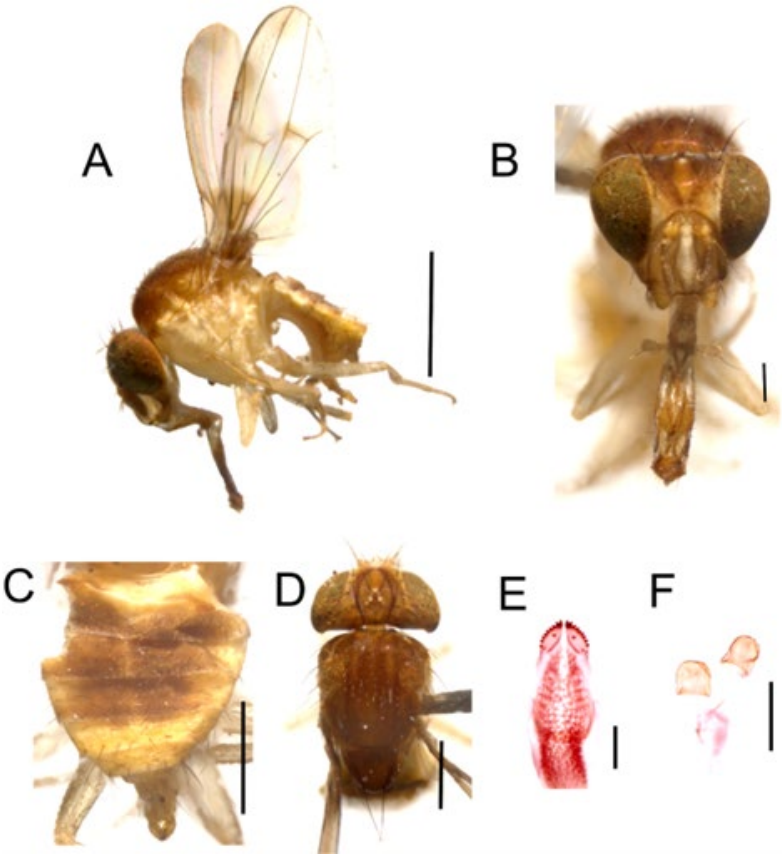


Figure 15. *Zygothrica neoaldrichi* ♀. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0,1mm).

Zygothrica cryptica Grimaldi (Figure 16)
Zygothrica cryptica Grimaldi, 1987: 216.

Type locality: Barro Colorado Island, Panamá.
Material examined: 01 ♂ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimen.
Geographical distribution: Brazil (State of Pará (Utinga, Belém do Pará) [new record]) and Panamá.

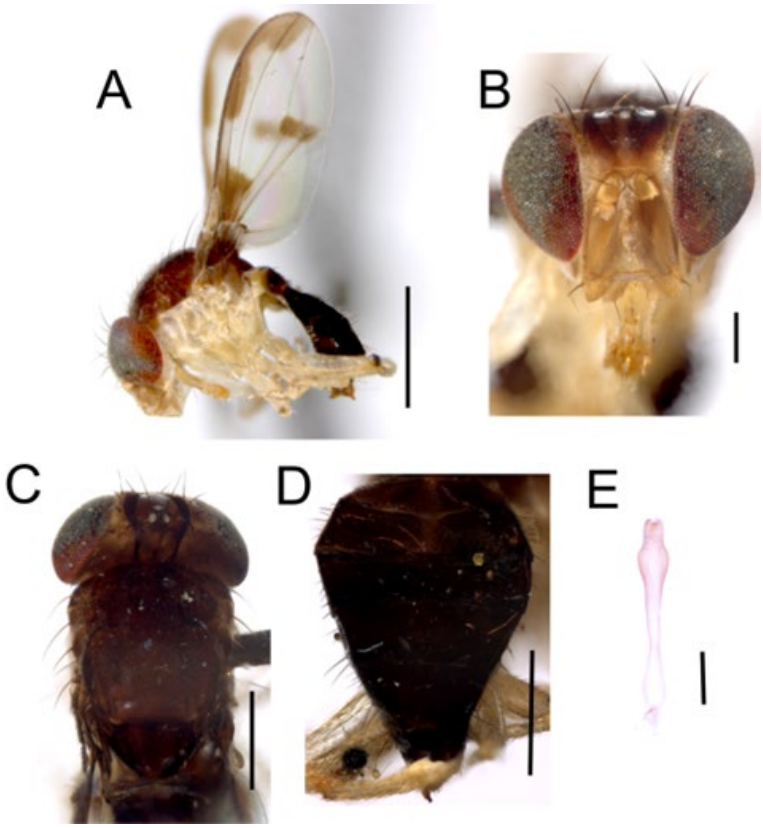


Figure 16. *Zygothrica cryptica* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: phallus and phallapodeme, dorsal view (scale bars: 0.1mm).

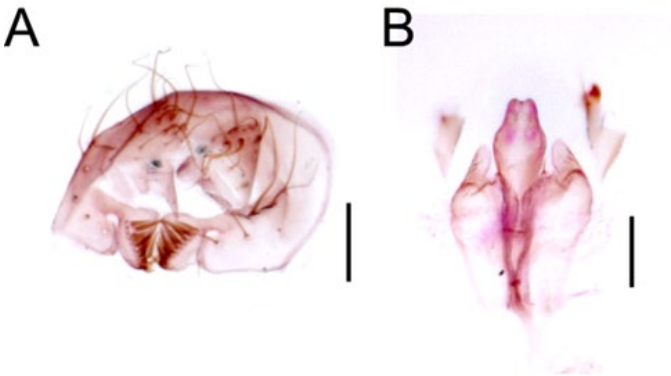


Figure 17. *Zygothrica nigropleura* ♂. A: epandrium, cerci, surstyli and subepandrial sclerite in posterior view; B: phallus, phallapodeme, hypandrium, postgonites, and pregonites in ventral view (scale bars: 0.1mm).

Zygothrica nigropleura Grimaldi (Figure 17)
Zygothrica nigropleura Grimaldi, 1987: 230.

Type locality: Salesópolis, São Paulo, Brazil.
Material examined: 01 ♂ labeled as "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japu[í]ba, L. Trav. F; VII.[1]936; Col: L. Trav". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimen.
Geographical distribution: Brazil (States of Santa Catarina, São Paulo and Rio de Janeiro (Angra dos Reis) [new record]).

Zygothrica paraldrichi Burla (Figures 18-19)
Zygothrica paraldrichi Burla, 1956: 223.

Type locality: Rio de Janeiro, Rio de Janeiro, Brazil.
Material examined: 06 ♂, 05 ♀ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes"; 01♂ labeled "Brasil, São Paulo, Ilha Seca, Com. IOC; 18/26.II.1940". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Brazil (States of Amazonas, Pará, Rio de Janeiro, Rondônia, and São Paulo), Guyana, Peru and Republic of Suriname.

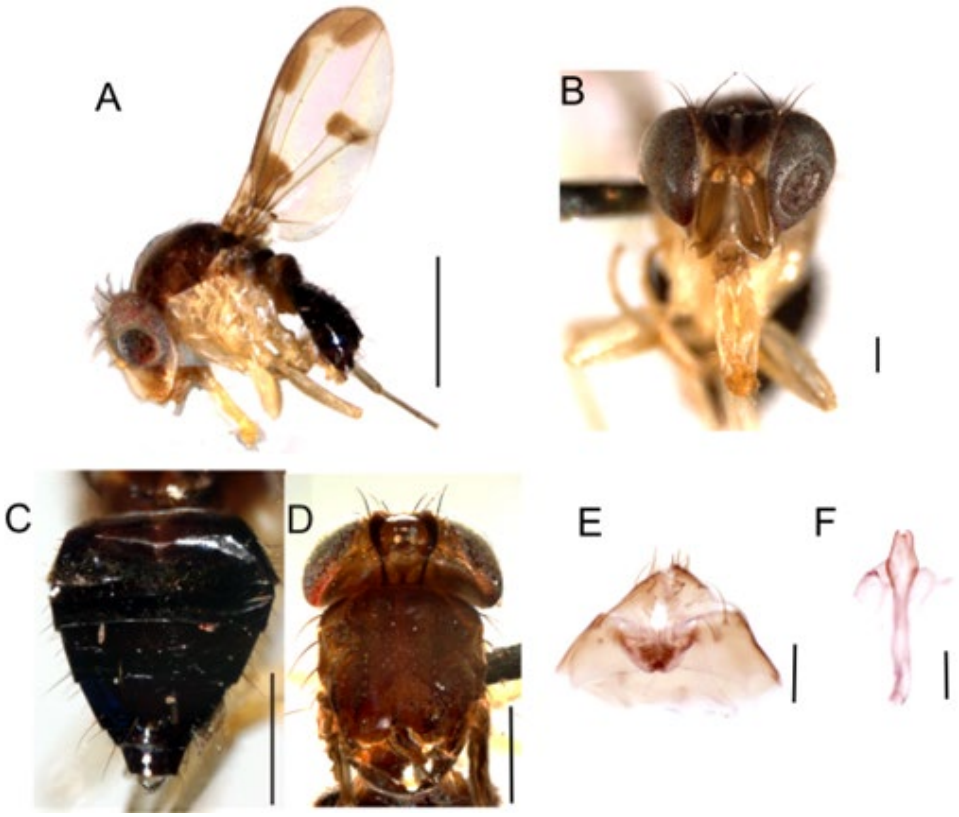


Figure 18. *Zygothrica paraldrichi* ♂. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view; (scale bars: 0.5mm; B: 0.2mm). E: epandrium, cerci, surstyli and subepandrial sclerite in posterior view; F: phallus and phallapodeme, ventral view (scale bars: 0.1mm).

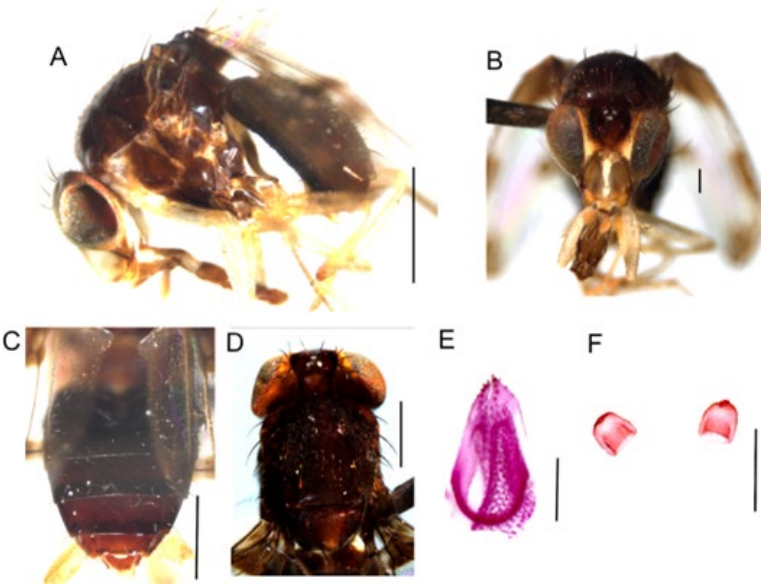


Figure 19. *Zygothrica paraldrichi* ♀. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0.1mm).

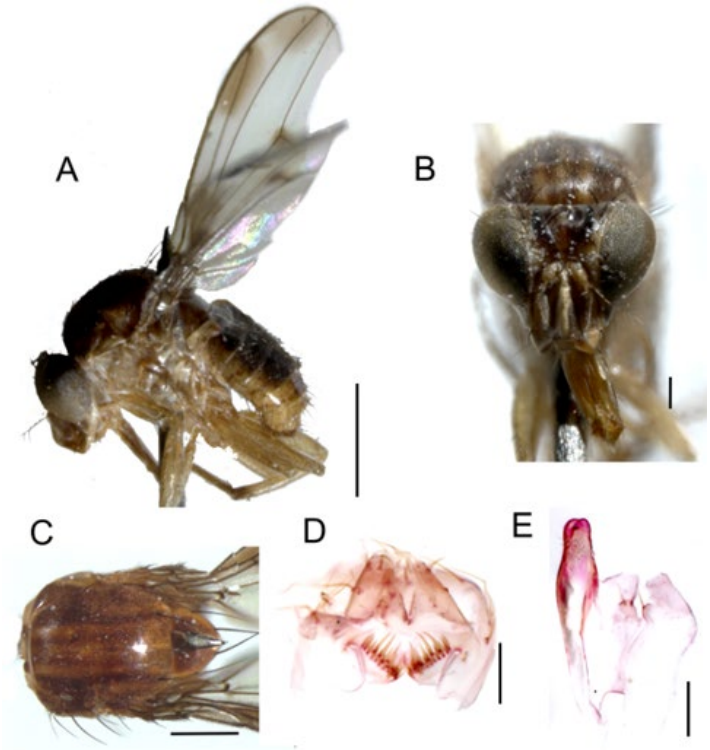


Figure 20. *Zygothrica somatia* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; (scale bars: 0.5mm; B: 0.2mm). D: epandrium, cerci, surstyli and subepandrial sclerite in posterior view; E: phallus and phallapodeme, ventral view (scale bars: 0.1mm).

Zygothrica somatia Grimaldi (Figure 20)
Zygothrica somatia Grimaldi, 1987: 236

Type locality: Monte Dourado, Pará, Brazil.
Material examined: 01 ♂ labeled "[Brazil], S[ão] Paulo, Cantareira, Horto Florestal; 3.[1]935; Col: L. Trav. F.". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimen.
Geographical distribution: Brazil (State of Pará and São Paulo [new record]).

Zygothrica zygia Grimaldi (Figure 21)
Zygothrica zygia Grimaldi, 1987: 209.

Type locality: Tabaquite, Trinidad.
Material examined: 01 ♂, 02 ♀ labeled "[Brazil], Go[i]as, [Goiânia]; 2.I.[1]936; Col: Borgmeier et S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographical distribution: Bolivia, Brazil (State of Amazonas, Goiás (Goiânia) [new record], Pará and Rondônia), Colombia, Guyana, Peru and Republic of Suriname.

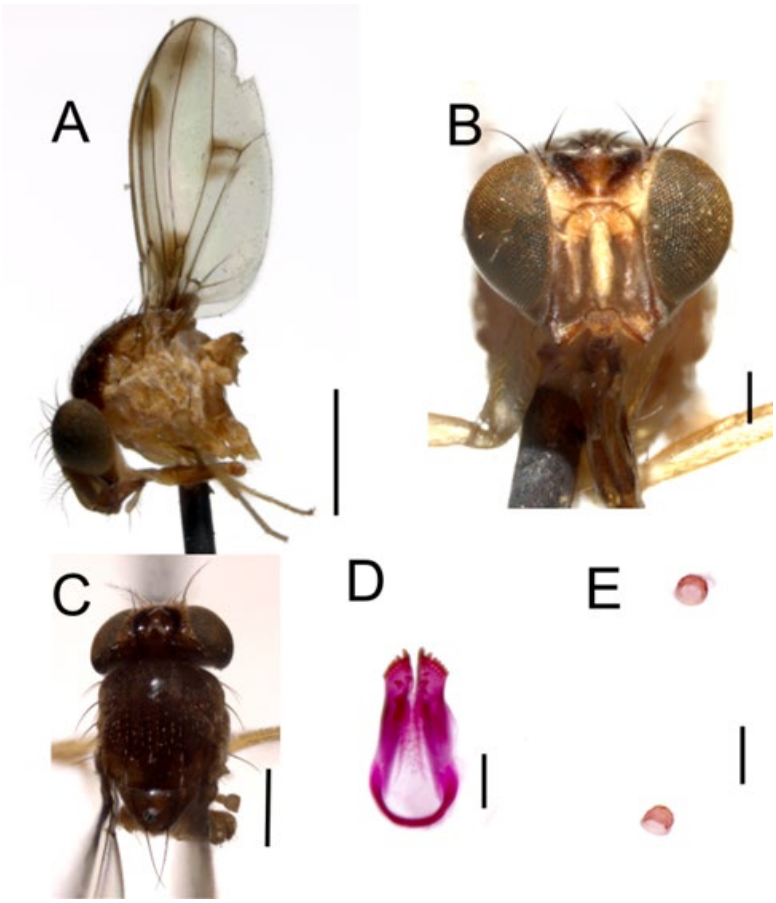


Figure 21. *Zygothrica zygia* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). D: oviscapt, ventral view; E: spermatheca (scale bars: 0,1mm).

virgatalba group

Zygothrica virgatalba Burla (Figures 22-23)
Zygothrica virgatalba Burla, 1956: 252.

Type locality: Rio de Janeiro, Rio de Janeiro, Brazil.
Material examined: 02 ♂, 01 ♀ labeled "Brasil, [Pará], Belém [do] Pará, Utinga; 22.VI.[19]65; Col: H. S. Lopes". 01 ♂ labeled "[Brazil], Go[i]as, [Goiânia]; 21.XII.[1]936; Col: Borgmeier et S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimens.
Geographic distribution: Brazil (States of Goiás [new record], Pará [new record], Rio de Janeiro and São Paulo).

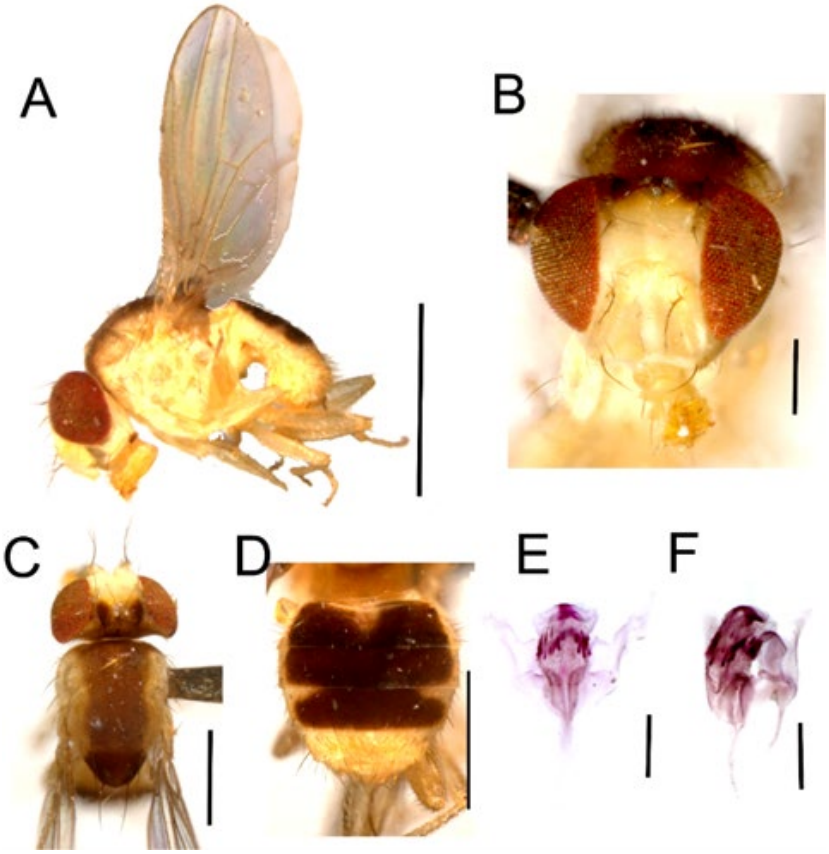


Figure 22. *Zygothrica virgatalba* ♂. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view; (scale bars: 0.5mm; B: 0.2mm). E: phallus and phallapodeme, ventral view; F: phallus and phallapodeme, lateral view (scale bars: 0.1mm). (scale bars: 0.1mm).

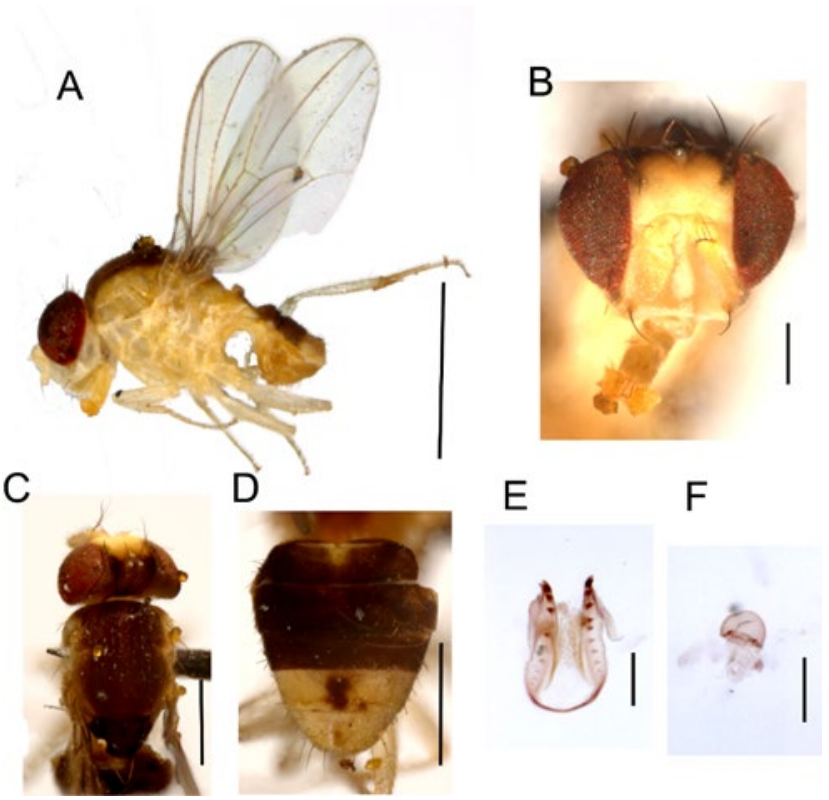


Figure 23. *Zygothrica virgatalba* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view; (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars: 0,1mm).

vittimaculosa group

Zygothrica vittinubila Burla (Figure 24)
Zygothrica vittinubila Burla, 1956: 225.

Type locality: Rio de Janeiro, Rio de Janeiro, Brazil.
Material examined: 01 ♀ labeled "[Brazil], E[stado] do Rio [de Janeiro], Angra [dos Reis], Japun[í]ba, L. Trav. F; VII.[1]936; Col: L. Trav." Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimen.
Geographic distribution: Brazil (States of Rio de Janeiro and São Paulo) and Peru.

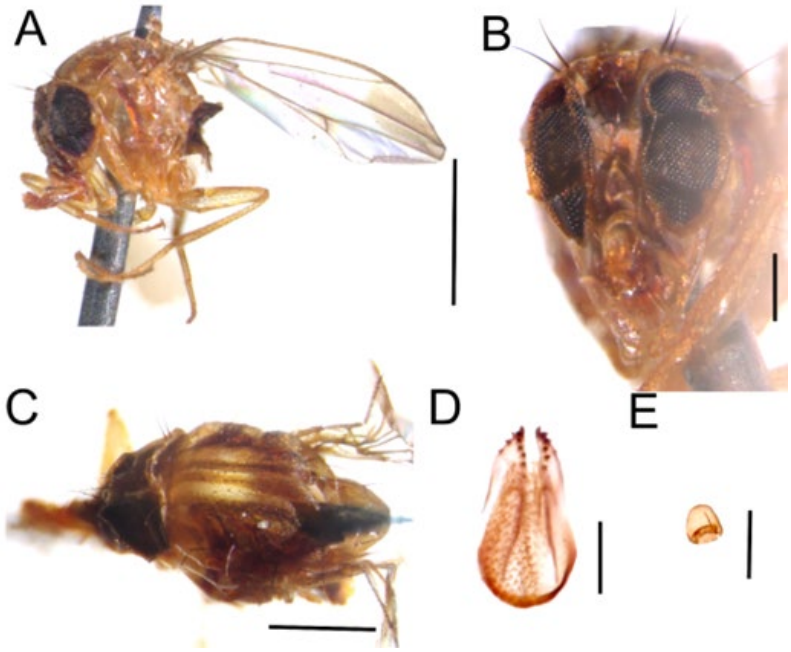


Figure 24. *Zygothrica vittinubila* ♀**.** A: lateral view; B: head, frontal view; C: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). D: oviscapt, ventral view; E: spermatheca (scale bars: 0.1mm).

Incertae sedis

Zygothrica vittipoecila Burla (Figure 25)
Zygothrica vittipoecila Burla, 1956: 231.

Type locality: Itatiaia, São Paulo, Brasil.
Material examined: 01 ♂ labeled "Brasil, E[stado] do Rio [de Janeiro], Petrópolis, Tq; II.[19]69; Col: H. S. Lopes". Abdominal sclerites and the terminalia were stored in microvials with glycerine and attached to the specimen.
Geographical distribution: Brazil (States of São Paulo and Rio de Janeiro [new record]).

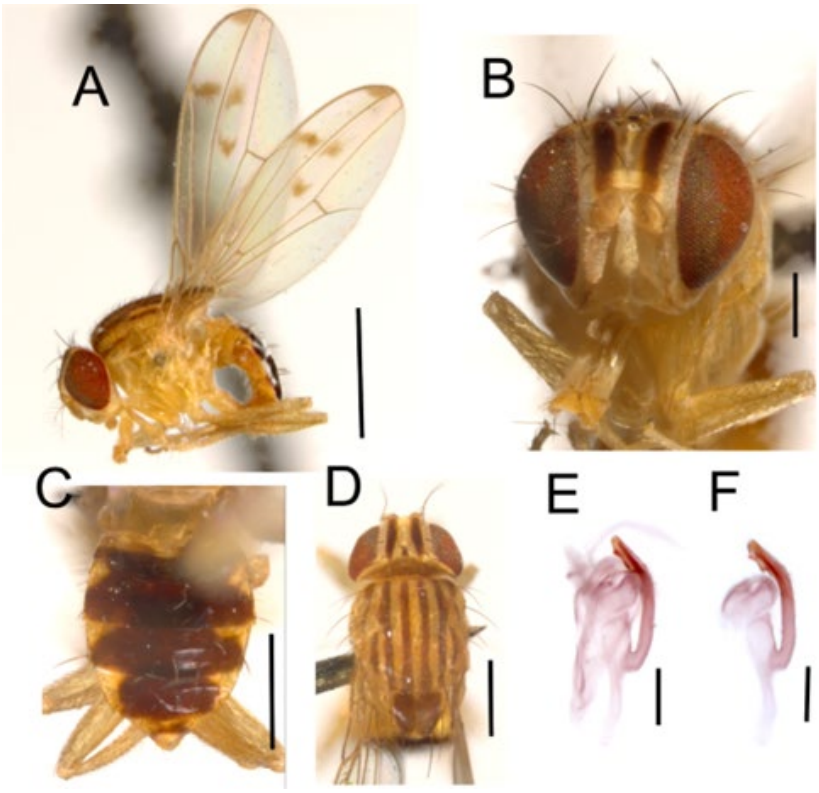


Figure 25. *Zygothrica vittipoecila* ♂. A: lateral view; B: head, frontal view; C: abdomen, dorsal view; D: thorax, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: phallus, phallapodeme, hypandrium, postgonites and pregonites in lateral view; F: phallus, phallapodeme, and postgonites in lateral view (scale bars: 0.1mm).

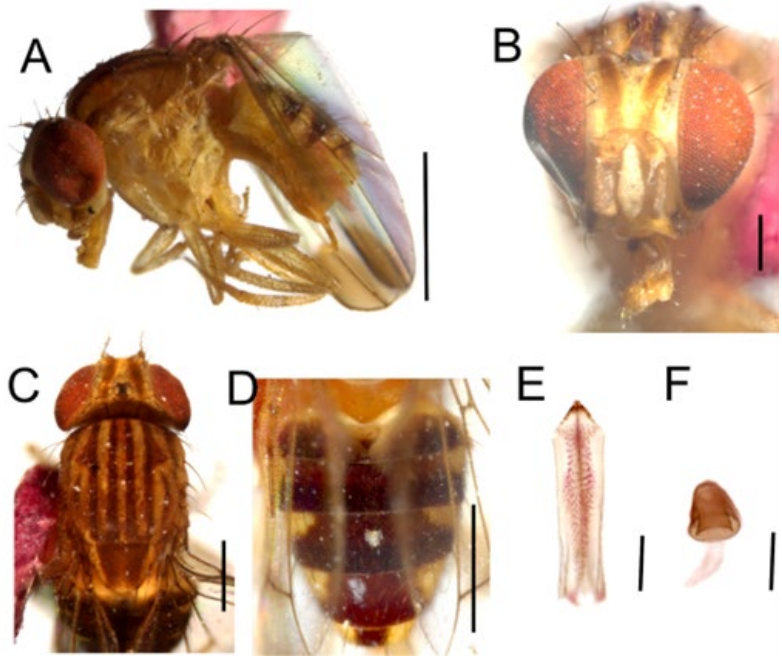


Figure 26. *Zygothrica vittisecta* ♀. A: lateral view; B: head, frontal view; C: thorax, dorsal view; D: abdomen, dorsal view (scale bars: 0.5mm; B: 0.2mm). E: oviscapt, ventral view; F: spermatheca (scale bars 0.1mm).

Zygothrica vittisecta Burla (Figure 26)
Zygothrica vittisecta Burla, 1956: 232.

Type locality: Itatiaia, São Paulo, Brasil.
Material examined: 01 ♀ labeled "Brasil, [Santa Catarina], Nova Teutônia; 27°11' B. 52° 23' L. 20.VI.1938; Col: Fritz Plaumann". 01 ♀ labeled "[Brasil], Alem Paraíba, Minas Gerais; 28.II.[19]48; Col: Cerqueira". Abdominal sclerites and the terminalia were stored in microvials with glycerin and attached to the material.
Geographical distribution: Brazil (States of São Paulo, Santa Catarina [new record] and Minas Gerais [new record]).

Discussion

Among the studied species, 15 represent new records of geographic distribution. Robe *et al*. (2014) expanded the geographic distribution of 16 southern and two northern *Zygothrica* species. Corroborating these results, we expanded the distribution records of *Z. bilineata*, *Z. dispar*, and *Z. prodispar* in areas where the models predicted its presence. The species *Z. nigropleura, Z. somatia, Z. virgatalba, Z. cryptica, Z. exuberans, Z. neoaldrichi, Z. aldrichi, Z. microeristes, Z. vittisecta, Z. vittipoecila,* and *Z. zygia* also had their distribution areas expanded (Bächli, 2020). In this study, the evaluated individuals were sampled up to the 60's, which indicates that this group is not widely sampled yet and, consequently, remains insufficiently investigated.

Acknowledgments; We thank Dr. Jane Costa, Dr. Márcio Felix, and MSc. Danielle Cerri from the Entomological Collection of the Instituto Oswaldo Cruz for allowing access to the specimens deposited in the museum. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under grant nº472973/2013-4 and n◦141578/2018-1.

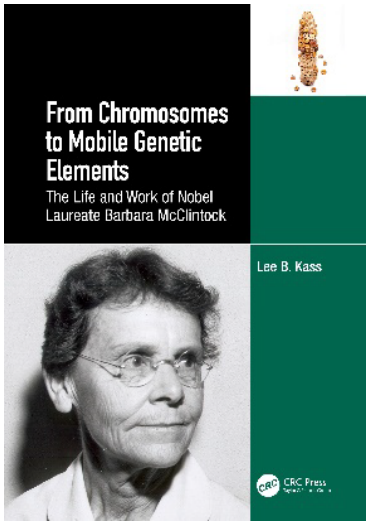
Availability of data and materials

In this study, we identify specimens collected in several localities from Brazil that were deposited from the Entomological Collection of Instituto Oswaldo Cruz (CEIOC), RJ, Brazil.

Ethics approval consent to participate

This research followed the guidelines specified by the research permits from the Entomological Collection of Instituto Oswaldo Cruz (CEIOC), RJ, Brazil.

References: Bächli, G., C.R. Vilela, A.S. Escher, and A. Saura 2004, Fauna Entomol. Scand. 39: 1-362; Bächli, G., 2022, TaxoDros: The database on taxonomy of Drosophilidae, v.1.04. Database, 2021; Burla, H., 1956, Mitt. Zool. Mus. Berl. 32: 189-321; Cumming, J.M., and D.M. Wood 2010, Ottawa: NRC Research Press. pp. 9-50; Grimaldi, D.A., 1987, Bull. Am. Mus. Nat. Hist. 186: 103-268; Grimaldi, D.A., 1990, Bull. Am. Mus. Nat. Hist. 197: 1-139; Gottschalk, M.S., P.R.P. Hofmann, and V.L.S. Valente 2008, Check List 4: 485-518; Mendes, M.F., and M.S. Gottschalk 2019, Rev. Bras. Entomol. 63: 250-253; Robe, L.J., S. Machado, A.R. Bolzan, J.P.J. dos Santos, F.B. Valer, A.P. dos Santos, M.L. Blauth, and M.S. Gottschalk 2014, Neotrop. Fauna E. 49: 79-94; Tidon, R., M.S. Gottschalk, H.J. Schmitz, and M.B. Martins 2017, Available in: http://fauna.jbrj.gov.br/fauna/faunadobrasil/183444 (accessed 26 October 2022).



A new biography of Nobel Laureate Barbara McClintock, by Lee B. Kass

A new biography of Nobel Laureate Barbara McClintock has been published by botanist and historian of science Lee B. Kass (2024). The book titled *From Chromosomes to Mobile Genetic Elements: The Life and Work of Nobel Laureate Barbara McClintock* can be ordered and previewed at the publishers website, CRC/Routledge/Taylor & Francis Group, www.routledge.com/9781032365329. The book ships free on every order across the globe.

The new biography of Nobel Laureate Barbara McClintock (1902-1992) places her life and work in its social, scientific and personal context. It examines the development of McClintock's scientific work and her influence upon individuals and upon the fields of cytogenetics and evolutionary biology in the period from 1902 to the present. The history documents years of McClintock's notable and lauded scientific work long before she discovered and named transposable elements in the mid-1940s for which she ultimately received the Nobel Prize. The biography employs documented evidence to expose, demystify, and provide clarity for legends and misinterpretations of McClintock's life and work.

Excerpt from the "Introduction":

> "This biography offers a different historical picture of McClintock's life and contributions, as previously reflected in published literature and legends. For example, readers will learn here that: McClintock was born in Hartford, Connecticut (not Brooklyn, NY); she participated in many extracurricular social and sports activities in high school (she was not antisocial or a loner); she graduated high school at age 17, in June 1919 (not mid-year at age 16); Professor Lester W. Sharp (not R. A. Emerson) was Barbara McClintock's major professor in graduate school; not until she had obtained her Ph.D. (not as an undergraduate) did she morphologically describe the ten chromosomes of corn (at mitosis, not meiosis); the first maize chromosome map was published in1922 by her genetics professor C. B. Hutchison (not by McClintock in 1931); she did not discover ring chromosomes, but reported finding the first *maize* ring chromosome in 1931; she was not denied tenure at the University of Missouri, but rejected a forthcoming offer of tenure and instead accepted a research position elsewhere; and Barbara McClintock is *not buried* at Huntington Rural Cemetery, on Long Island, NY, etc. These and many other legends will be demystified in the chapters that follow" (Kass 2024, p. 1).

Kass, L. B. 2024. *From Chromosomes to Mobile Genetic Elements: The Life and Work of Nobel Laureate Barbara McClintock* (1st ed.). CRC/Routledge/Taylor & Francis Group. www.routledge.com/9781032365329

An updated perspective on Hermann Joseph Muller's Nobel Prize winning research: An unshared award

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Abstract

This perspective reminds readers of Nobel Laureate H. J. Muller's early publications and announcements that led to his 1946 Nobel Prize award for the discovery of X-radiation effects and induced mutations and brings to the reader's attention the limitations of the Nobel award process. It briefly discusses the process of publications and review during the early years of the 21st century, and offers commentary on how the definition of mutation has changed over time, by citing a few reports showing that Muller's hypothesis that X-rays lead to "point mutations" may have been documented and that his thoughts on low-dose radiation threshold remain controversial.

Introduction

Articles questioning the scientific review process, priority and results for H. J. Muller's 1946 Nobel Prize winning research, in Physiology or Medicine, "for the discovery of the production of mutations by means of X-ray irradiation" (Nobel Prize 1946) and arguments that Muller had made a critical mistake by reporting that he had discovered X-ray induced "gene" mutation, which allegedly led him to the wrong evaluation of the Linear No-threshold Theory (LNT) of radiation exposure for cancer risk (e.g. Calabrese 2011, 2017, 2018), had inspired my earlier assessment of those assertions (Kass 2018). In this perspective I provide a revised, updated and further documented evaluation of those allegations.

This perspective I) again reminds readers of Muller's early publications and announcements that led to his 1946 Nobel Prize award for the discovery of X-radiation effects and induced mutations and brings to the reader's attention the limitations of the Nobel award process; II) briefly discusses the process of publications and review during the early years of the 21st century; and III) offers commentary on how the definition of mutation has changed over time, by providing a few reports showing that Muller's hypothesis that X-rays lead to "point mutations" may have been documented and reveals that his thoughts on low-dose radiation threshold – evaluation of the Linear No-threshold Theory (LNT) of radiation exposure for cancer risk assessment – remain controversial. Yet, recent research provides additional confirmation for Muller's warnings of the dangers of low-dose ionizing radiation.

Ia. Muller's 1927/1928 announcements on X-radiation and mutation

Priority in scientific discovery traditionally has been determined by dates of article submission, therefore many journals provide the submission date at the time of publication. Often such papers require revisions and dates of publication may be delayed a year or more after the first submission date. A new discovery might occasionally first appear as an abstract given at a conference, recognized as a brief report in a scientific journal, and then followed elsewhere by a longer, more detailed publication. Currently, one might also provide the date of first online appearance prior to the official publication date.

Hermann J. Muller, then at the University of Texas, published, on July 22, 1927 in the journal *Science* a brief four page paper titled "Artificial Transmutation of the Gene" (Muller 1927a). In Berlin, about two months later, on Thursday, 15 September 1927, at the 5th International Congress of Genetics (5th ICG, 11-18 September; Nachtsheim 1928: 47-48) as an invited speaker (Anonymous 1927a; Nachtsheim 1927, pp. 5, 9), Muller presented extensive data to support his evidence. This pioneering discovery was immediately heralded around the world (Serebrovsky 1927, Anonymous 1927b, Gates 1927). Muller's report, including methods and data tables, but without a literature cited, was published the following year (March 1928) in the *5th ICG Proceedings* (Muller 1928b, Kass 2024: 47). Curt Stern (1974: 29) recalled that Muller arrived in Berlin with only a very rough draft of his Congress paper, which was typed up only after he arrived. Stern also made the

diagrams of the crosses that Muller presented at the Congress (Stern was in charge of photographs and "episcopic projections" for presentations; Nachtsheim 1928: 8). Other presenters also brought only "drafts" of their papers, for which editor Hans Nachtsheim (1928) offered grateful thanks in his foreword (*Vorwart*) to the published Congress *Proceedings*. Contributors had been requested to submit their manuscripts "to the Secretary, Dr. H. Nachtsheim, before the opening of the Congress;" the ultimate date for receipt of manuscripts was September 17 (see "The Fifth International Genetics Congress" 1927: 210). Furthermore, each lecturer was asked to "prepare separately a short abstract of his paper and hand it to the Secretary before or at the opening of the Congress."

Hans Nachtsheim was more than qualified to review drafts and edit the *Proceedings* for the 5th ICG. He was affiliated with Erwin Baur's Institute for Heredity at the Agricultural College in Berlin-Dahlem (Nachtsheim 1928, Plarre 1987), and had spent the academic year 1926-1927 in T. H. Morgan's lab at Columbia University on an International Education Board Fellowship sponsored by the Rockefeller Foundation (The Rockefeller Foundation 1972: 220; Harwood 1993: 39; Deichmann 1996: 232). Stern too was well qualified to review Muller's paper after spending two years (1924-1926) in Morgan's lab learning *Drosophila* genetics (Stern 1974; The Rockefeller Foundation 1972: 303). We can expect that they would have had no difficulties accepting, rejecting, or suggesting revisions to Muller's draft manuscript.

Additionally, Nachtsheim had earlier published a German translation of Morgan's (1919) *Physical Basis of Heredity* (Morgan 1921; Deichmann 1996: 231). Morgan had sent a signed copy to R. A. Emerson, Chair of Plant Breeding at Cornell, which was later sent to the Cornell Archives for their History of Science collection.

Geneticist Lewis J. Stadler, then at the University of Missouri, conducting similar studies in plants, did not attend the 1927 International Congress of Genetics, in Berlin. He was scheduled to present a paper (Anonymous 1927a; Nachtsheim 1927, p. 10), but his name does not appear on the list of attendees in the *5th ICG Proceedings* (Nachtsheim1928). It is possible that had he been there to give his paper, his discovery of X-rays and mutation in barley and maize, simultaneously and independently of Muller, would have been remembered and noted by a Nobel Committee 19 years later, and perhaps have resulted in a shared award.

Before his paper appeared in the 1928 Congress *Proceedings*, Muller gave a presentation and published an abstract on his X-ray work at the American Association for the Advancement of Science (AAAS) meeting, in the Joint Genetics Section of the American Society of Zoologists and The Botanical Society of America in Nashville, Tennessee, on Wednesday, 28 December 1927 (Muller 1927b: 174). This is actually Muller's 3rd contribution on X-ray findings during 1927: First in July, *Science;* and second, in a presentation September 1927 at ICG Berlin, which was subsequently published in the March 1928 *5th ICG Proceedings* (Muller 1928b).

For his contribution "read" before the Joint Genetics Sections at the December 1927 Nashville meeting, Muller (1927b) was awarded the annual prize of the AAAS for his paper titled, "Effects of X-Radiation on Genes and Chromosomes," as announced in the January 27th, 1928 issue of *Science*:

> "The American Association prize of $1,000 is awarded annually to the author of a notable contribution to the advancement of science given at the annual meeting. The funds are generously supplied by a member who wishes his name withheld. Nominations for the Nashville prize were received from the secretaries of the sections and societies and the award was made by the committee on prize award and announced through the news service Friday evening.
>
> The winner of the prize is this year Dr. H. J. Muller, professor of zoology in the University of Texas, for his outstanding contribution entitled "Effects of X-Radiation on Genes and Chromosomes," which was presented before the Joint Genetics Section of the American Society of Zoologists and the Botanical Society of America, in the Wednesday-morning session. The following abstract of Dr. Muller's paper has been contributed by him, having been sent from Austin, Texas, by air mail" (Livingston 1928a: 81-82).

Muller's (1928a) "more detailed" invited abstract dated January 8, 1928 was published in the same *Science* issue to accompany the announcement by Permanent Secretary Burton E. Livingston, of the Annual AAAS

Prize, the fifth to be awarded by the American Association for the Advancement of Science, and immediately follows his announcement.

Names of the awarding committee are listed below Muller's (1928a) *Science* abstract; Two were members of the National Academy of Sciences [NAS], William Duane Prof. of Biophysics, Harvard [NAS elected 1920], and Charles Schuchert, Professor of Paleontology, Yale University [NAS elected 1910]. Three other committee members were Robert J. Terry (Chair) Professor of Anatomy, Washington University, St. Louis; L. J. Cole, Professor of Genetics, University of Wisconsin, and G. Canby Robinson Professor of Paleontology, Yale University, New Haven, Connecticut.

Three months later, at the end of April 1928, Muller again presented his research to a meeting of the National Academy of Sciences in Washington, DC. His 12 page published paper, including references, was titled, "The Production of Mutations by X-Rays." It provided the details of the report for which he won the AAAS award in Nashville, in addition to more recent results, and appeared five months later in the September Academy *Proceedings* (Muller 1928c).

Since Muller was not yet a member of the NAS, his paper first had to be reviewed by members of the Academy (see Part II, below), which may have delayed immediate publication. I could find no documentation of who invited Muller to present his work to the NAS, but it may have been T. H. Morgan (NAS elected 1909), with whom Muller studied for his Ph.D. between 1912 and 1915 (degree awarded 1916) at Columbia University (*Catalogue 1916-1917*: 281). Or possibly, one of the NAS members, who was on the committee that awarded the AAAS Prize at the 1927 Nashville meeting, had extended the invitation.

Muller's (1928c) report to the NAS, clearly recognized that he, in addition to others, had been conducting research on the "effectiveness of X-rays in producing both gene mutation and chromosome reorganization in Drosophila." L. J. Stadler, in particular was lauded for similar studies in plants:

> "Stadler, working on barley and maize, has conclusively demonstrated, by means of ingenious methods, both gene mutations and chromosome aberrations … to be produced in plants by x-rays. It should be stated, in this connection, that his work was carried on simultaneously with and independently of that of the present writer" (Muller 1928c: 722).

Muller (1928c) lists publications by Stadler, and others, to support his accounts, although he does not cite specific papers within this text by author and date, as one commonly does in scientific publications today. Only two of three Stadler publications, listed by Muller, can be found in the literature (Stadler 1928a,b). One paper that Muller misdated as "Stadler, L. J. 1928a" and titled "Genetic Effects of X-Rays in Maize and Barley," is listed by Muller as an "address to Genetics Section, A.A.A.S., Dec[ember], 1927; title in *Anat. Rec*., 37, [p.] 176." It is actually an unpublished abstract by Stadler (1927, see below). Muller also misdated his own abstract and address [presentation] as "1928a", for his contribution published for that same December 1927 meeting – the presentation for which he was awarded the AAAS annual prize (Muller 1927b; see above).

I searched all the titles and abstracts in *The Anatomical Record* Volume 37, December 1927, and found Muller's abstract, #134, on page 174 (see Muller 1927b). But I could neither locate a title, nor an abstract for Stadler on page 176 (or elsewhere), as listed by Muller in his 1928c *PNAS* paper. I began to think that Muller might be mistaken, when my colleague from University of Missouri brought to my attention that Stadler (1928b) began his August *Science* article on mutation in barley by reminding readers of his contribution at the December 1927 AAAS meeting in Nashville: "At the Nashville meeting of the American Association last December I reported the occurrence of mutations in barley following X-ray treatment." And that Marcus Rhoades (1956) had also reported on Stadler's participation at that event. Stadler (1928b) cited no abstract for his participation at the AAAS meeting, but his presentation is mentioned in a National Academy memoir by Rhoades (1957). No abstract dated December 1927, however, accompanies the list of publications enumerated by Rhoades. Confirmation of Stadler's presentation, however, can be found in the report of the Secretary for the Joint Genetics Sections held at the AAAS meetings in Nashville, December 1927, which was published in *Science,* 3 February 1928 (Dunn 1928: 125; Livingston 1928b). The report summarizes papers given at the Joint Genetics Sections at the 1927 Nashville meeting of the AAAS, and mentioned the contributions by both Muller and Stadler:

> "Forty contributions were offered, twenty-four of them being read at the formal sessions –Five of the papers read, one by demonstrations, and one of the papers given by title only, dealt with the effect of X-rays on plants and animals. Chief interest centered in the recent attempts to alter the course of inheritance and the frequency of mutation by treatment with X-rays. The most extensive experiments on the question were reported in detail by H. J. Muller, whose paper (for which the American Association Prize was awarded this year) is abstracted in the section on the Prize. By use of a special technique for measuring the frequency of mutations in *Drosophila melanogaster* he obtained results indicating that the application of sublethal doses of X-rays to sperm was followed by a large increase in the mutation rate of treated as compared to control, cultures. The mutation rate in some treated cultures was estimated at 15,000 times the normal rate. … From the botanical side L. J. Stadler reported on the occurrence of new endosperm characters that apparently had arisen by mutation in maize ears X-rayed at the time of fertilization. The same investigator presented evidence for the occurrence of mutations in seedlings from treated barley seeds" (Dunn 1928: 125).

Secretary Dunn's report certainly confirms both Stadler's recollection and Muller's misdated-citing of Stadler's presentation at the AAAS meeting in December 1927.

It is worth mentioning that Stadler announced his X-ray results, not unlike Muller, as (1) a contributed paper to the Joint Genetics Section of the AAAS in December 1927, (2) a publication in *PNAS* (January 1928, submitted in November 1927), and (3) a paper in *Science* (August 1928). Any argument regarding Muller publishing in these venues to avoid peer review (Calabrese 2018), could also be applied to Stadler (and others). But as I will demonstrate below (Part II), Muller's more detailed reports on X-rays and mutation *were* reviewed by his peers, and his first announcement in *Science* was the appropriate venue for the time.

Stadler, Muller, and his collaborator Edgar Altenburg at the Rice Institute, continued this topic at the Joint Genetics Section AAAS meeting held in New York City, 28 December 1928 (*The Anatomical Record* 1928: 88, 97, 100). The session was chaired by Muller and included Stadler (1928c, abstract #12, p. 97), who reported that the "rate of mutation … varies in direct proportion to the intensity of irradiation." Muller and Altenburg (1928, abstract #19, p. 100) reported that "frequency of translocations … produced by X-rays … was found to arise with nearly the frequency of detectable gene mutations." Clearly, Muller and Stadler were both continuing to study "gene" mutation and chromosomal rearrangements induced by X-rays (see Muller & Altenburg 1930).

Ib. Limitations of the Nobel Awards Process

Many would agree that both Muller and Stadler should have shared the Nobel Prize for the "discovery of the production of mutations by means of X-ray irradiation." Note that the term mutation has changed over time (see below). But many people who should have shared the prize have not—it is influenced by many factors and in some cases this award is quite political, as was show by Istvan Hargittai in his 2002 book titled *The Road to Stockholm.* The decisions are dependent on the nominations (and nominators-see Hargittai 2002: 22, for the six groups who may nominate for physiology or medicine prize), and the members of the Swedish Academy who review them. Hargittai (2002: Chapters 3 & 12) elucidates why some received the prize and others have not. He explains that the review process is a considerable task that is bound to produce mistakes, since the committees are not and have not been the most informed on the weight of discoveries in subfields in which they may have no extensive experience. They very much rely on the information submitted by the nominators. Kass (2024: Chapter 11) has an extensive discussion on this topic.

Although Hargittai makes no mention of Stadler with respect to Muller's unshared 1946 Nobel Prize, we know that Stadler was using X-rays for studies of plant mutations as early as 1926 (or earlier), as was Muller in *Drosophila*, and Muller (1928c) gave him credit for such in his *PNAS* article *and* years later in his Nobel Prize Lecture:

> "And Stadler, in his great work on the production of mutations in cereals, started independently of our own, has obtained evidence that in this material X-radiation in the doses used is unable to produce a sensible rise in the gene mutation frequency, though numerous chromosome breakages do arise,

> leading to both gross and minute rearrangements of chromosome parts. Either the genes are more resistant in this material to permanent changes by X-rays, as compared with their responsiveness to thermal agitation, or a break or loss must usually be produced by X-rays along with the gene change" (Muller 1946).

Clearly here Muller was recalling Stadler's later studies on X-rays inducing chromosomal rearrangements, and not his earliest results, seemingly similar to Muller's own, on gene mutation.

Muller's understanding of a gene was technically a locus on a chromosome and his point mutation was a place on that locus. Carlson (1991, 2013), Muller's former student and his biographer, explained that the definition of the gene, and by extension a gene mutation, was an evolving concept (see part IIIa, below). Since in 1946, no one really knew what a gene was at that time, and since Muller (1928c) also included translocations, inversions etc. in the "production of mutations by X-rays," the sharing of the prize with Stadler might have been appropriate.

Another view of the politics of the Nobel Prize is offered in a book by Friedman (2001). Even though it focuses on the prizes in chemistry and physics, it depicts how the members of the committees make decisions on who gets a Nobel Prize, and how many of those judgements included political, national, self-interest, as well as resentful, negotiated, and closed-minded agendas. It shows extensive interpersonal clashes between committee members and how downplaying of important nominees and their works was a frequent practice. Norrby (2010: 17-21) and Kass (2024: 232) provide additional clarification on the selection process.

The files on Nobel Prize winners are closed for 50 years (Tønnesson 1999; Hargittai 2002: 16), and Muller's would have been available to researchers in 2006. Perhaps answers to some of these procedures clarifying the committee's decision for an unshared prize in Physiology or Medicine for 1946 will be found therein?

II. Process of publications and review during the early years of the 21st century

Publishing in *Science*—speedy publication:

It has been argued that Muller circumvented the review process in order to claim priority for his discovery of mutations induced by X-irradiation (Calabrese 2018). As mentioned above, Muller (1928c) promptly and directly credited Stadler for simultaneously and independently conducting similar studies in plants. Moreover, brief announcements of new discoveries were routinely published in *Science* and in *Nature* with an expectation that details would be forthcoming elsewhere. An historical view of this protocol is well presented by Baldwin (2014, 2015a,b; 2018).

Baldwin (2014) elucidates that "many of the most influential texts in the history of science were never put through the peer review process, including Isaac Newton's 1687 *Principia Mathematica*, Albert Einstein's 1905 paper on relativity, and James Watson and Francis Crick's 1953 *Nature* paper on the structure of DNA." And academic journals trusted … "Prominent scientists on their editorial boards to make decisions about which papers to print." The term "peer review" she informs, originated after World War II. Using examples from *Nature*, Baldwin explains that the scientific weekly *Nature*, did not consult referees for every paper it printed until 1973.

Specifically, she uses the example of Watson and Crick's 1953 *Nature* paper, announcing the structure of DNA and for which they ultimately won a Nobel Prize in Medicine or Physiology. Their paper, she explains (Baldwin 2015a), serves as a useful illustration of two important features of publications in *Nature*: first, its reputation for relatively speedy publication, and second, the extent to which the editors relied on prominent scientists, particularly British ones, to recommend content. *Nature* was "known as a venue for the fast publication of new results in the early twentieth century," and both Watson and Crick wanted a note that could be published quickly.

I believe that in the United States, an analogy could be made for the journal *Science*. By examining the list of papers provided in the *Biographical Memoirs* of the National Academy of Sciences, one can see new and exciting "firsts" published in *Science* by its membership. For examples, in addition to Muller and Stadler, one will find papers published in *Science* by future Nobel Laureates T. H. Morgan and by Barbara McClintock

to quickly announce new and exciting research during the 1920s and earlier (see McClintock 1929, Sturtevant 1959, Kass 2013ff.; Kass 2024: 62). Furthermore, only one month after Watson and Crick had announced their DNA structure in *Nature*, Stanley Miller—then a graduate student of 1934 Nobel Laureate Harold Urey—announced in a two page technical paper in *Science* his famous experiment producing organic molecules in an atmosphere replicating that of primitive Earth (Miller 1953, Bada & Lazcano 2007). Arguably, *Science* had continued to be the best venue in the United States to promptly announce new discoveries.

Publishing in *PNAS*—guidelines for review

As I mentioned above, Muller's presentation to the 5th ICG in Berlin was in all probability reviewed and edited by Hans Nachtsheim (1928). Furthermore, as a nonmember of the Academy, Muller's 1928c *PNAS* paper, presented in April and published in September *required* review, as per NAS Constitution and Bylaws (Cochrane 1978a,b):

> "OF SCIENTIFIC COMMUNICATIONS, PUBLICATIONS AND REPORTS
> XV. Papers from persons not members, read before the Academy, Classes or Sections, and intended for publication, shall be referred at the meeting at which they are read, to a Committee of members competent to judge whether the paper is worthy of publication. Such Committees shall report to the Academy as early as practicable, and not later than the next stated session. If they do not then report, they shall be discharged, and the paper referred to another Committee" (Cochrane 1978b: 612).

The publication of Muller's presentation to the NAS had been delayed by five months (Muller 1928c). Most likely it had been reviewed by a "Committee," that included Morgan, who became NAS President in 1927, among others of the NAS section to which his paper was presented in April of 1928.

Similarly, Stadler's (1928a) *PNAS* paper, published in January, had been communicated to the NAS the previous November (1927), most likely by E. M. East (NAS elected 1925), and also would have *required* review. Stadler, who worked with East as a National Research Council (NRC) Fellow at Harvard during 1925-1926, was not a NAS member (until elected in 1938). Emerson, too, having been elected to the NAS in 1927, likely reviewed Stadler's (1928a) *PNAS* paper, and Muller's paper as well. And as Chair, Emerson presumably had invited Stadler to present his X-ray work at the Joint Genetics Section in Nashville. It is not clear why Stadler's abstract was not included in the *Anatomical Record*. Emerson was well acquainted with Stadler's research, as the latter did part of his NRC fellowship in Emerson's department at Cornell during 1925-1926 (Kass 2005; Kass 2024: 36; *Synapsis Club Record Book 1925-1926*; Rèdei 1971: 6).

Coe and Kass (2005) reminded readers that *PNAS* was a forum to get new results published quickly and was not supposed to include all details of the investigations. Articles were originally limited to 6 pages and occasionally went over this limit when funds were available; the editors wanted some evidence of how the work was obtained but elaborate tables, graphs and the description of details were specifically prohibited, but exceptions could be made. The editor explained that around 1925-1926, a special grant became available to enlarge the size of the *Proceedings* and take some articles in excess of 6 pages provided they were still short. By 1933 the funds had been spent and they had to return to the old rule of the *Proceedings* to limit articles to 6 pages (until recently). Indeed, Creighton and McClintock's pioneering report on crossing-over in maize was broken up into two papers (McClintock 1931, Creighton & McClintock 1931) following sequentially in the journal, and issued as one reprint (Coe & Kass 2005; Kass 2013ff.: 1.123-1.145; Kass 2024: Chapter 5).

Stadler's (1928a) *PNAS* paper was limited to 6 pages, but Muller's (1928c) paper was twice that and possibly required special permission for the excess pages. No tables or graphs are included in Muller's September publication, but he did list a paper he submitted on 25 October of 1927, and published 1 July 1928, volume 13, issue 4 of *Genetics*–which included in its 78 page report, an extensive list of references and many tables. The periodical was known at the time to be a refereed journal, and then published bi-monthly.

Without doubt, papers by nonmembers of the NAS, who published in *PNAS*, were definitely reviewed prior to their publication, as per NAS Constitution and By-laws (Cochrane 1978a,b).

IIIa. Definitions of genes and mutations have changed over time

An argument that Muller had not found that X-rays affect point-mutation seems unrealistic (Calabrese 2018) when one did not know what a point-mutation was in 1946 (Kass 2018; Kass 2024: 21, 79, 105). Carlson (1991) provides a summary and excellent chronological table showing how the term gene and gene mutation has changed over time. More recently Portin and Wilkins (2017) and Norrby (2022) provide additional perspectives on the changing concept of the gene. Overlooked by Carlson, but credited in *The search for the gene* published by the *Drosophila* geneticist and NAS member Bruce Wallace (1992), are the contributions of Alfred Hershey and Martha Chase (1952) to the confirmation of the gene as DNA. These researchers working in the Department of Genetics, Carnegie Institution of Washington, at Cold Spring Harbor, Long Island, New York, "are often credited with having performed *the* experiment that finally located the gene, thus ending the long search," even though it was inconclusive "in the view of the authors" (Wallace 1992:115, 117). This was of course seven years after Avery et al. submitted their report in November of 1943, published in 1944, demonstrating that genes (the heredity material) were DNA. Max Delbrück, Alfred D. Hershey and Salvador E. Luria shared The Nobel Prize in Physiology or Medicine 1969 "for their discoveries concerning the replication mechanism and the genetic structure of viruses"; Oswald Avery, who died in 1955, was no longer eligible to be considered for the award (Hargittai 2002: 226).

Knowing that the hereditary material was DNA still did not permit an understanding of the physical limitation of the gene on the chromosome. Studies led to the knowledge that point mutations are changes in the sequence of DNA bases, and include substitutions, insertions, and deletions of one or more nucleic acid bases. Many of these were shown to be caused by chemical mutagenic agents, but ionizing radiation was also believed to play a part in such phenomena (Lehninger 1975: 881).

When Muller studied genes and mutations, "mutation" was understood to be a sudden, hereditary change in the genetic makeup of an organism. Simply defined, the term mutation can be of two types: gene mutations or point mutations, and chromosomal mutations (Gleason 2017; see also any modern textbook of Genetics). Gene mutations include local changes in the structure or composition of genes whereas chromosomal mutations or chromosomal aberrations involve large changes in the structure (e.g., inversions and translocations) or number of chromosomes.

In 1946, Muller's contribution to understanding genes and mutations was limited by our knowledge of the gene at that time. Modern technology has permitted more refinement of how structural and functional genes are studied (see below).

IIIb. Muller's hypothesis that X-rays may lead to "point mutations"

After the initial work, Stadler continued studies of mutations expanding to work with induced and spontaneous mutations. Stadler eventually argued that X-rays may be removing genes rather than changing genes (Stadler 1954; Kass 2024: 165-166). Stadler and Muller debated this issue (cordially) for some years.

As mentioned above, mutations can be defined as either point mutations or chromosomal mutations (Carlson 1991). Part of the argument in favor of X-rays causing point mutations was that reverse mutations could be caused by X-rays. A popular textbook of the time classified mutations as changes in the chromosome and as changes in the composition of individual genes (Sturtevant & Beadle 1939: 206). By 1939, doubts raised that "*x*-rays do not induce gene mutations, but only cause breakage and reunion of chromosomes," was believed to have been resolved, by producing reverse mutations (wildtype to mutant, and mutant back to wildtype) by X-rays in the *forked* locus in *Drosophila*. By citing work of drosophila geneticists (Muller, Patterson and Timofeeff-Ressovsky), Sturtevant and Beadle (1939: 215) reported that this result "leaves little doubt that actual gene changes are concerned."

This was still the prevailing belief when Muller was awarded his Nobel Prize in 1946 (see Sinnott, et al. 1950: 257, 291). Decades later, in a new Preface to the reprint of their original textbook, Sturtevant and Beadle (1962: 9) acknowledged "changes that a thorough revision would need to incorporate …". They specifically emphasize topics in "Chapter XIII … discussion of the mechanism of the induction of mutations by irradiation needs revision, and the chemical induction of mutations would play a large part in an up-to date account." Shortly thereafter, the second edition of the *General Genetics* textbook coauthored by Beadle's

former student, Adrian Srb (et al. 1965: 244ff.), included a section on induced mutation for a consideration of ionizing radiation, and chromosomal vs. gene mutations.

Years later, after insertions of transposable elements (transposons) were recognized to change the action of genes, and long after Barbara McClintock was awarded the 1983 Nobel Prize "for her discovery of mobile genetic elements" (Kass 2024: Chapter 11), it was shown that the *forked* allele is due to insertion of *gypsy*, an LTR-retrotransposon (long terminal repeat transposon, Kuzin et al. 1994). This means that the reversion in *forked* could be due to a small deletion of part or the entire element, rather than a point mutation (J. Birchler, pers. com, 11 Dec. 2018).

More elaborate results on mutation frequency were show by Timofeeff-Ressovsky in the 1930's with alleles of the *white* locus in *Drosophila*—presenting data of x-ray dosage with increased gene mutation at the *white* locus, his results showed that "different genes, even different alleles at a single locus, may have quite different mutation rates" (Sturtevant & Beadle 1939: 215-217). Mel Green conducted X-ray reversion studies on alleles of *white*, *yellow* and *scute* (Green 1961), and also found some were capable of being reverted by X-rays while others were not. There is a perfect correlation of those revertible alleles being retrotransposon insertions and those that were refractory to reversion, having no major restriction fragment alterations. This result is consistent with X-rays causing small deletions (of the retrotransposon to restore lost gene function).

Studies of the "white gene" in *Drosophila* (Mackenzie et al. 1999) apparently used an X-ray induced allele and a spontaneous mutation to study partially pigmented eye color mutant strains of *D. melanogaster. The study reports DNA sequence results on "*the nature and location of the *point mutations* which identify functionally important regions of the *D. melanogaster* guanine and tryptophan ABC transporters." The case they described might be a bona fide case of a point mutation induced by X-rays (Kass 2018).

Jim Birchler (U of Missouri, pers. com. 30 November 2018) wrote to me that he has worked with *white-coffee* (and *satsuma*) of the *white* locus. It could well be a point mutation generated by X-rays. However, he also noted he had recovered transposon induced mutations from [chemical mutagen] EMS treatments, and he noted … it is always the case that, when you are looking for something, whatever you find may or may not be caused by the applied agent but instead be "spontaneous". Indeed, the case of *satsuma* illustrates that "point mutations" can be spontaneous.

I fully concur with Jim's comment, "Whether X-rays mainly delete genes or change genes or both does not detract from the fact that the ability to produce alterations of genes rather than relying on spontaneous mutations was a milestone in the history of the field of genetics."

IIIc. Is Muller's correlation of no threshold for cancer risk still controversial?

In a workshop on Radiation and Cancer, Muller's early research on the danger of low-dose X-radiation was reviewed, along with the correlation that ionizing radiations may also have a carcinogenic potential. Citing the work that questioned Muller's low-dose radiation studies with respect to cancer (Calabrese 2011), Magrini (2015) summarized epidemiologic studies in Japan on the effects in humans exposed to the atomic bomb. He stated, "It is not possible to distinguish the tumors possibly caused by radiation, morphologically, from "naturally" occurring cancers. … The dose effect curve generally rises more steeply with high-LET [Linear Energy Transfer] radiation than with low-LET, especially at low dose rates (the reduction in radiation dose-response at high doses is consistent with a cell killing effect)."

Magrini explains that study of cancers caused by radiation exposure has been the "subject of a vast amount of scientific contributions ... Yet, the issue is largely an unresolved one ... and it is charged with a strong 'emotional' content. The consequences of under-or overestimating this problem may be equally dangerous and costly."

Regarding the potential risks of "low radiation doses," he continues, "… the theory holds that excess cancer risks related to low-dose radiation are directly proportional to the dose. Despite some controversy over the excess cancer risk of low-dose radiation, the linear no-threshold theory [LNT] is widely used because an alternative method for assessing the potential risks of low-dose radiation is lacking. Some [researchers] question the validity of the linear no-threshold theory and think that below a certain threshold carcinogenesis ceases to be a concern" (see e.g., Calabrese 2017).

Magrini further cautions, “Although the estimated risks from low levels of radiation of a single CT (computer assisted tomography) exam are uncertain, it is prudent to minimize the dose from CT by applying common sense solutions and using other simple strategies as well as exploiting technologic innovations. These efforts will enable us to take advantage of all the clinical benefits of CT while minimizing the likelihood of harm to patients.” In a published literature review and consensus statement, his group (Buglione et al. 2016) subsequently counseled: with regard to “Radio-therapeutic precautions, … no clear thresholds have been reported in the literature … It is therefore desirable to reduce the dose [for treatment].”

Do more recent studies of correlations of low does radiation and cancer uphold Muller’s warnings?

The U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute, reported a study demonstrating that low dose radiation has been linked to leukemia. Investigators in their Radiation Epidemiology Branch and colleagues from other institutions, led by senior investigator Mark Little, Ph.D. were able to quantify—for the first time—excess risk for leukemia and other myeloid malignancies following low-dose exposure to ionizing radiation in childhood (Little et al. 2018a).

A second report, published in the *European Journal of Epidemiology,* identified a significant exposure-response between low-dose cumulative occupational radiation exposure to the eye lens and risk of cataracts, based on self-reported history (Little et al. 2018b). More recently these researchers have analyzed dose response for cancer and cardiovascular disease risks in populations exposed to ionizing radiation and quantified radiation-cancer dose-response relationship at low and very low doses. A recent report showed that low dose radiation is linked with elevated risk of cardiovascular disease (Little et al. 2023). Additional studies demonstrate that data may be affected by many variables (Little et. al. 2024).

As emphasized by Haynes (1998) Muller’s discovery sensitized him immediately to the health hazards of the careless or excessive use of diagnostic X-rays in medical practice, and later of radioactive fallout from nuclear weapons tests. It may have taken a long time to prove, but the studies cited above may help resolve the controversy regarding the dangers of low, in addition to, high dose exposure to X-rays over time.

CONCLUSIONS

The answer to the query, “Was Muller’s 1946 Nobel Prize research for radiation-induced gene mutations peer reviewed?” is: 1) Yes, as per the accounts and documents presented here, 2) but may not be an appropriate question to ask for the time period.

Historical perspective demands that we interpret reviews of Muller’s Noble Prize publications in the context of the times, and not in light of current academic guidelines (Kass 2003: 1255; Kass 2024: Chapter 6). Upon H. J. Muller’s retirement from Indiana University in 1964, his colleague Professor Tracy Sonneborn (1964) paid tribute:

> “Mankind will long continue to profit by his personal contact, lectures and writings in the years ahead.”

Following Muller’s death three years later, Professor Guido Pontecorvo (1968) praised how Muller’s “decisive contributions – both in theory and in experiments, many of them in advance of his time – opened and marked step by step the trail from the Mendelism of the 1910's to the molecular biology of the 1960’s.”

Additionally, as geneticist and historian of science Bentley Glass (1990) reminded us, “Let us grant that personal emotions and failures of memory may obtrude in such documents” as testimonies written years after the events. “It is of course the historian's mission to check all conflicting evidence against other records, and so attempt to ferret out the truth.” Reading the memories that accompany Glass’ (1990) article, certainly demonstrates that recollections must be documented by contemporaneous records in order for truth to emerge (see Kass 2024: 131, 249).

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Annotated References Cited:

Anonymous. 1927a. The Fifth International Genetics Congress, Program of General Session for the week; Americans contributing to the congress. *Science,* New Series 66(No. 1705, Sep. 2), p. 210. [listed as a contributor is "L. J. Stadler, Columbia University" (*sic*; Stadler was at University of MO); also accurately reports that Muller presents his address on Thursday, Sept. 15].

Anonymous. 1927b. X-Rays and Evolution "Science News" Sep. 16, 1927, Supplement to *Science* 66(No. 1707), pp. x-xiv. [Report on Muller's X-ray paper delivered the previous day, at the 5th ICG, 11-18 September 1927, Berlin].

Avery, O. T., C. M. MacLeod, M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus Type III. *Journal of Experimental Medicine* 79(No. 2, Feb. 1): 137–158 [Received: November 1, 1943], https://doi.org/10.1084/jem.79.2.137

Bada, J. and A. Lazcano. 2007. Stanley L. Miller, 1930-2007, A Biographical Memoir. *Biographical Memoirs*, National Academy of Sciences, Washington, D.C.

Baldwin, M. 2014. Essay: Did Isaac Newton need peer review? Scholarly journals swear by this practice of expert evaluation. But It's a new phenomenon that isn't the only way to establish the facts. *Zócalo*, April 29, 2014. http://www.zocalopublicsquare.org/2014/04/29/did-isaac-newton-need-peer-review/ideas/nexus/

Baldwin, M. 2015a. Credibility, peer review, and *Nature*, 1945–1990. Notes Records, *Royal Society of London* 69(No. 3, Sep. 20): 337–352. [Published Online: 1 July 2015] https://doi.org/10.1098/rsnr.2015.0029

Baldwin, M. 2015b. *Making "Nature"*: *The History of a Scientific Journal*, University of Chicago Press (18 August 2015).

Baldwin, M. 2018. Scientific autonomy, public accountability, and the rise of "Peer Review" in the Cold War United States *Isis* 109(No. 3, Sep.): 538-558.

Buglione, M., R. Cavagnini, F. Di Rosario, et al. 2016. Oral toxicity management in head and neck cancer patients treated with chemotherapy and radiation: Xerostomia and trismus (Part 2). Literature review and consensus statement. *Critical Reviews in Oncology/Hematology* 102(June): 47-54. http://dx.doi.org/10.1016/j.critrevonc.2016.03.012

Calabrese, E. J. 2011. Review: Muller's Nobel lecture on dose–response for ionizing radiation: ideology or science? *Archives of Toxicology* 85(No. 12, Dec.): 1495–1498. [Online: 30 June 2011]

Calabrese, E. J. 2017. Review: Flaws in the LNT single-hit model for cancer risk: An historical assessment. *Environmental Research* 158(Oct.): 773-788. [Online: 27 July 2017]

Calabrese, E. J. 2018. Review: Was Muller's 1946 Nobel Prize research for radiation-induced gene mutations peer reviewed? *Philosophy, Ethics, and Humanities in Medicine* 13(June 6): 6 [5 pp.]. https://doi.org/10.1186/s13010-0 18-0060-5

Carlson, E. A. 1991. Defining the gene: An evolving concept. *American Journal of Human Genetics* [*AJHG*] 49 (No. 2, Aug.): 475-48.

https://europepmc.org/backend/ptpmcrender.fcgi?accid=PMC1683308&blobtype=pdf

Carlson, E. A. 2013. Reflections in Mutation Research: H.J. Muller's contributions to mutation research. *Mutation Research/Reviews in Mutation Research* 752(No. 1, January–March): 1-5. https://doi.org/10.1016/j.mrrev.2012.08.003

Catalogue 1916-1917, *Columbia University in the City of New York*. Degrees conferred 1915-1916, Doctors of Philosophy, Hermann Joseph Muller, A.B. 1910, A.M. 1911, Major subject: Zoology, conferred June 7th 1916, pp. 280-282.

Cochrane, R. C. 1978a. *The National Academy of Sciences: The first Hundred Years 1863-1963.* National Academy of Sciences, Washington, D.C.

Cochrane, R. C. 1978b. Appendix C. Constitution and By-Laws of the National Academy of Sciences, Adopted January 1864, ARTICLE XV. Of Scientific Communications, Publications and Reports, p. 612. *In, The National Academy of Sciences*: *The first Hundred Years 1863-1963.* National Academy of Sciences, Washington, D.C.

Coe, E. and L. B. Kass. 2005. Proof of physical exchange of genes on the chromosomes. *Proceedings of the National Academy of Sciences* 102(No. 19, May 10): 6641-6656. [Online: May 2, 2005] http://www.pnas.org/content/102/19/6641.full [Reprinted in Kass 2013ff.]

Creighton, H. B. and B. McClintock. 1931. A correlation of cytological and genetical crossing-over in *Zea mays*. *Proceedings of the National Academy of Sciences* 17(No. 8, 15 August, communicated 7 July 1931): 492–497. [Communicated the same date and issued as one reprint with McClintock 1931; Reprinted in Kass 2013ff.]

Dunn, L. C. 1928. Organizations Related to Sections F and G [Zoology and Botany]. *In,* The Genetics Sections of the American Society of Zoologist and the Botanical Society of America. Chairman R. A. Emerson, Secretary/treasurer L. C. Dunn. *Science* 67(No. 1727, Feb. 3): 125.

Deichmann, U. 1996. *Biologists Under Hitler,* Translated by Thomas Dunlap. Harvard University Press (5 May 1999, 468 pp.).

Friedman, R. M. 2001. *Politics of Excellence: Behind the Nobel Prize in Science***.** W H Freeman & Co., New York (October 1, 2001).

Gates, R. R. 1927. International Congress of Genetics. *Nature* 120(No. 3022, Oct. 1): 495-496. https://doi.org/10.1038/120495a0

Glass, B. 1990. The grim heritage of Lysenkoism: Four Personal Accounts, I. Foreword. *Quarterly Review of Biology* 65(No. 4, Dec.): 413-421. [An introduction to four memoirs of events by those who were present during the 1930s and 1940s]

Gleason, K. M. 2017-03-07. Hermann Joseph Muller's Study of X-rays as a Mutagen, (1926-1927). https://embryo.asu.edu/pages/hermann-joseph-mullers-study-x-rays-mutagen-1926-1927

Green, M. M. 1961. Back mutation in *Drosophila melanogaster*. I. X-ray induced back mutations at the Yellow, scute and white loci. *Genetics* 46(No. 6, June, received 17 Jan.): 671-682.

Haynes, R. H. 1998. Heritable variation and mutagenesis at early International Congresses of Genetics, *Genetics* 148(No. 4, April): 1419-1431.

Hargittai, I. 2002. *The Road to Stockholm*: *Nobel Prizes, Science, and Scientists.* Oxford University Press. Oxford, UK.

Harwood, J. 1993. *Styles of Scientific Thought; The German Genetics Community, 1900–1933*, The University of Chicago Press, Chicago, IL.

Hershey, A. D. and M. Chase. 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *The Journal of General Physiology* 36(No. 1, Sep. 20, received 9 April): 39–56.

Kass, L. B. 2003. Records and recollections: A new look at Barbara McClintock, Nobel Prize-Winning geneticist. *Genetics* 164(No. 4, Aug. 1): 1251–1260. https://www.genetics.org/content/164/4/1251 [PDF available here: https://pmc.ncbi.nlm.nih.gov/articles/PMC1462672/]

Kass, L. B. 2005. Missouri compromise: tenure or freedom. New evidence clarifies why Barbara McClintock left Academe. *Maize Genetics Cooperation Newsletter* 79(Oct. 31): 52-71. [Online: April 2005] https://mnl.maizegdb.org/mnl/79/05kass.htm

Kass, L. B. (ed.). 2013ff. *Perspectives on Nobel Laureate Barbara McClintock's publications (1926-1984): A Companion Volume*. Volumes I & II The Internet-First University Press. URI: http://hdl.handle.net/1813/34897 [Online: Dec. 30, 2013; updates March 2014; Vol. III, Increments No. 1-3, May 2016; Vol. IV, Increment No. 4, April 2020.]

Kass, L. B. 2018. Questions and reflections on H. J. Muller's Nobel Prize winning research. *Drosophila Information Service* 101(Dec.): 93-106. https://www.ou.edu/journals/dis/DIS101/DIS101.pdf

Kass, L. B. 2024. *From Chromosomes to Mobile Genetic Elements: The Life and Work of Nobel Laureate Barbara McClintock* (1st ed.). CRC/Routledge/Taylor & Francis Group. www.routledge.com/9781032365329

Kuzin, A B, N V Lyubomirskaya, B M Khudaibergenova, Y V Ilyin, and A I Kim.1994. Precise excision of the retrotransposon gypsy from the *forked* and *cut* loci in a genetically unstable *D. melanogaster* strain. *Nucleic Acids Research* 22(No. 22, Nov. 11): 4641–4645. https://doi.org/10.1093/nar/22.22.4641

Lehninger, A. L. 1975. *Biochemistry. The molecular basis of cell structure and function,* 2nd ed. Worth Publishers, Inc.

Little, M. P., R. Wakeford and D. Borrego, et al. 2018a. Leukaemia and myeloid malignancy among people exposed to low doses (<100 mSv) of ionising radiation during childhood: a pooled analysis of nine historical cohort studies. *Lancet, Haematology* 5(No. 8, Aug.): E346-E358 [Online: 16 July 2018]. https://doi.org/10.1016/S2352-3026(18)30092-9

Little M. P., C. M. Kitahara, E. K Cahoon, et al. 2018b. Occupational radiation exposure and risk of cataract incidence in a cohort of US radiologic technologists. *European Journal of Epidemiology* 33(No. 12, Aug. 27): 1179–1191. https://doi.org/10.1007/s10654-018-0435-3

Little, M., T. B. Azizova, D. B. Richardson, et al. 2023. Ionising radiation and cardiovascular disease: systematic review and meta-analysis. *British Medical Journal* [*BMJ*] 380: e072924, March 8, 2023, https://doi.org/10.1136/bmj-2022-072924

Little, M. P., M. Boerma, M-O Bernier, et al. 2024. Effects of confounding and effect-modifying lifestyle, environmental and medical factors on risk of radiation-associated cardiovascular disease. *BMC Public Health*

24(15 June 2024): [Number] 1601. https://bmcpublichealth.biomedcentral.com/articles/10.1186/s12889-024-18701-9
[Title Abbreviation for *BioMed Central public health*; See additional papers on this topic at: https://dceg.cancer.gov/about/staff-directory/little-mark]

Livingston, B. E. (ed.) 1928a. Special Issue containing General Reports of the Second Nashville Meeting of the American Association for the Advancement of Science and Associated Societies. Edited by Burton E. Livingston, Permanent Secretary. *Science* 67(No. 1726, Jan. 27): 77-85.

Livingston, B. E. (ed.) 1928b. Special Issue Containing Reports of the Sessions of Sections and Societies at the Second Nashville Meeting of the American Association for the Advancement of Science and Associated Societies. Edited by Burton E. Livingston, Permanent Secretary. *Science* 67(No. 1727, Feb. 3): 113-131.

Mackenzie, S. M., M. R. Brooker, T. R. Gill, G. B. Cox, A. J. Howells, G. D. Ewart. 1999. Mutations in the white gene of *Drosophila melanogaster* affecting ABC transporters that determine eye colouration. *Biochimica et Biophysica Acta* (BBA) – *Biomembranes*. 1419 (No. 2, July 15): 173-185.

Magrini, S. M. 2015. Potential risks of «low» radiation doses for the patients and the public. Congressi AIRO, I° AIRB COURSE IN RADIOBIOLOGY, Brescia Meetings in Radiation Oncology, 2015 Edition, The Power of Biology, Brescia, October 8th-9th 2015.
https://www.congressiairo.it/brescia-meetings-in-radiation-oncology-2015-edition-the-power-of-biology/

McClintock, B. 1929. Chromosome morphology in *Zea mays*. *Science* 69(No. 1798, 14 June 1929): 629. [The first published ideogram of *Zea* chromosomes; Reprinted in Kass 2013ff.]

McClintock, B. 1931. The order of the genes *C*, *Sh*, and *Wx* in *Zea mays* with reference to a cytologically known point in the chromosome. *Proceedings of the National Academy of Sciences* 17(No. 8, 15 August, communicated 7 July): 485–491. [Communicated the same date and issued as one reprint with Creighton and McClintock 1931; Reprinted in Kass 2013ff.]

Miller, S. 1953. A production of organic compounds under possible primitive Earth conditions. *Science* 117(No. 3046, 15 May, received 15 Feb.): 528-529.

Morgan, T. H. 1919. *The Physical Basis of Heredity*. Monographs on Experimental Biology, J. B. Lippincott Co. Philadelphia and London.

Morgan, T. H. [H. Nachtsheim, Translator]. 1921. *Die stoffliche Grundlage der Vererbung von Thomas Hunt Morgan* ... mit 118 Abbildungen. Vom verfasser autorisierte deutsche Ausgabe, von Hans Nachtsheim ... (English translation given as *Physical Basis of Life*, German) [Morgan's book was published as *Physical Basis of Heredity* in 1919.] Berlin, Gebrüder Bortraeger, 1921. https://newcatalog.library.cornell.edu/catalog/1161895

Muller, H. J. 1927a. Artificial transmutation of the gene. *Science* 66(No. 1699, July 22): 84-87.
[Reprinted in Muller 1962: 245-251]

Muller, H. J. 1927b. Effects of x-radiation on genes and chromosomes. Abstract #134. *In*, Program and Abstracts for the twenty-fifth annual meeting of the America Society of Zoologists, *The Anatomical Record* 37(No. 2, Dec.): 174 (see also p. 115, List of Titles, Genetics, Read, "134. Effects of x-radiation on genes and chromosomes, H. J. Muller, University of Texas (also by demonstration)." [Muller (1928c) misdated this abstract as published 1928.]

Muller, H. J. 1928a (January). Abstract: Effects of x-radiation on genes and chromosomes. *Science* 67(No. 1726, Jan. 27): 82. [Reported under "The Fifth Award of the American Association Prize" (pp. 81-83), see Livingston 1928a; Reprinted in Muller (1962: 276-277, 593) with the date January 8, 1928, exactly as it appeared in *Science*.]

Muller, H. J. 1928b. The problem of genic modification. *In*, Nachtsheim, Hans (ed.). 1928 [March]. *Proceedings of the 5th International Congress of Genetics, Berlin, 1927*, Vol. 1. [*Verhandlungen des v. Internationalen Kongresses für Vererbungswissenschaft, Berlin, 1927,* hrsg. von Hans Nachtsheim] *Zeitschrift für induktive Abstammungs- und Vererbungslehre*; suppl. 1: 234–260. [Often cited only by supplement title; reprinted in Muller 1962: 252-276.] [Cited by Muller (1928c) with a 1927 publication date.]

Muller, H. J. 1928c. The production of mutations by X-rays. *Proceedings of the National Academy of Sciences* 14(No. 9, presented 24 April, published 1 Sept.): 714-726. ["read before the Academy April 24th 1928" (U.S. National Academy of Sciences)]

Muller, H. J. 1946. The production of mutations, Nobel Lecture, December 12, 1946 https://www.nobelprize.org/prizes/medicine/1946/muller/lecture/ (accessed at Nobel Media AB 2018. Fri. 30 Nov 2018).

Muller, H. J. 1962. *Studies in Genetics, The selected papers of H. J. Muller.* Bloomington, Indiana University Press.

Muller, H. J. and E. Altenburg. 1928. Abstract #19. Chromosome translocations produced by x-rays in *Drosophila*. *The Anatomical Record* 41(No. 1, 25 Dec.): 100. [see also p. 88]

Muller, H. J. and E. Altenburg. 1930. The frequency of translocations produced by X-rays in Drosophila. *Genetics* 15 (No. 4, July 1): 283–311, https://doi.org/10.1093/genetics/15.4.283 [PDF available here: https://pmc.ncbi.nlm.nih.gov/articles/PMC1201066/ ; cites Muller & Altenburg. 1928. Chromosome translocations produced by X-rays in Drosophila. (Abstract) *Anat. Rec*. 41:100]

Nachtsheim, H. 1927. *Fünfter Internationaler Kongress für Verbungswissenschaft* Berlin, 11-17 September 1927 [Program and Abstracts]. Leipzig, Verlag von Gebrüder Borntraeger, 1927. https://link.springer.com/content/pdf/10.1007/BF01959591.pdf

Nachtsheim, H. (ed.). 1928. *Proceedings of the 5th International Congress of Genetics, Berlin, 1927* [*Verhandlungen des v. Internationalen Kongresses für Vererbungswissenschaft, Berlin, 1927,* hrsg. von Hans Nachtsheim] *Zeitschrift für induktive Abstammungs- und Vererbungslehre*; suppl. 1-2; March 1928, date in foreword. [now available Online: https://catalog.hathitrust.org/Record/102847298]

Nobel Prize. 1946. The Nobel Prize in Physiology or Medicine. 1946. https://www.nobelprize.org/prizes/medicine/1946/summary/
(accessed at Nobel Prize Outreach AB 2024. Mon. 1 Jul 2024).

Norrby, E. 2010. *Nobel Prizes and Life Sciences*, World Scientific Publishing Co. Pte. Ltd, Singapore.
[24 September 2010]

Norrby, E. 2022. The changing concept of the gene through the lens of Nobel Prizes awarded in the past century. Office of NIH History lecture series, Monday, February 13, 2023, 11:00:00 AM, https://videocast.nih.gov/watch=49150

Plarre, W. 1987. A contribution to the history of science of heredity in Berlin, pp. 147–218, *In*, H. Scholz (ed.), *Botany in Berlin, Englera 7*. Botanischen Garten und Botanischen Museum, Berlin-Dahlem.

Pontecorvo, G. 1968. Hermann Joseph Muller. 1890-1967. *Biographical Memoirs of Fellows of the Royal Society* 14(November 1968): 348-389, https://www.jstor.org/stable/769450 [Accessed: 22 December 2018 18:30 UTC]

Portin, P. and A. Wilkins. 2017. Perspective: The evolving definition of the term "Gene". *Genetics* 20(No. 4, April 1): 1353–1364, https://doi.org/10.1534/genetics.116.196956

Rèdei G. P. 1971. A portrait of Lewis John Stadler. 1896-1954, pp. 5–20, *In*, G. P. Redei and G. Kimber (eds.), *Stadler Genetics Symposia* (1969 & 1970) Vols. 1 & 2, University of Missouri, Agricultural Experiment Station, Columbia, MO.

Rhoades, M. M. 1956. L. J. Stadler. *Genetics* 41(No. 1, January 1956): 1-3. [Reports that Stadler gave a presentation at the Nashville meeting of the AAAS in 1927; not available in digital format].

Rhoades, M. M. 1957. Lewis John Stadler, 1896—1954. A Biographical Memoir. *Biographical Memoirs*, National Academy of Sciences, Washington, D.C.

Serebrovsky, A. S. 1927. Four pages that shook the world. *Pravda*, 11 Sept. 1927 [Alexander Sergeevich Serebrovsky, soviet geneticist], as reported in Stark, A. and E. Seneta. 2011. A.N. Kolmogorov's defense of Mendelism. *Genetics and Molecular Biology* 34(No. 2, April-June): 177–186. [Online: Apr. 1, 2011] [Formerly *Brazilian Journal of Genetics*; Last edition of journal was published 2016; Currently only available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3115669/]

Sinnott, E. W., L. C. Dunn, and Th. Dobzhansky. 1950. *Principles of Genetics* (4th ed.). McGraw-Hill Book Co. Inc., NY. [The change in the definition of the terms genes and mutations can be followed through the years by examining previous editions of this book. The first edition was published in 1925; the 5th and last edition published in 1958.]

Sonneborn, T. 1964. Hermann Joseph Muller. https://biology.indiana.edu/documents/historical-materials/Muller_Hermann_retirement.pdf [Accessed: 8 July 2024]

Srb, A., R. Owen, R. S. Edgar. 1965. *General Genetics*, 2nd ed. W. H. Freeman and Co. San Francisco & London.

Stadler, L. J. 1927, unpublished abstract. "Genetic effects of X-rays in maize and barley," presentation at Joint Genetics Sections AAAS December 1927. [Muller (1928c) cited this title but misdated Stadler's unpublished abstract as "Stadler, L. J. 1928a. Dec 1927; title in *Anat. Rec*., 37, [p.] 176"; Note that no title or abstract by Stadler is included in *The Anatomical Record* volume 37 (Dec. 1927); However, see Dunn 1928, p. 125, for his report of Stadler's presentation.]

Stadler, L. J. 1928a. Genetic effects of X-rays in maize. *Proceedings of the National Academy of Sciences* 14(No. 1, 15 January; communicated 23 Nov. 1927): 69-75.

Stadler, L. J. 1928b. Mutations in barley induced by X-rays and radium. Special Articles. *Science* 68(No. 1756, 24 August 1928): 186-187.

Stadler, L J. 1928c. Abstract #12. The rate of induced mutation in relation to dormancy, temperature, and dosage. *The Anatomical Record* 41(No. 1, 25 Dec.): 97 [see also p. 88].

Stadler, L. J. 1954. The gene. *Science* 120(No. 3125, 19 November 1954): 811–819. [Presidential Address to *American Society of Naturalists*, 30 December 1953; Published posthumously November 1954]

Stern, C. 1974. Presidential Address: "The Domain of Genetics." *Genetics* 78(No. 1, 1 September 1974): 21-33 [Not available Online]

Sturtevant, A. H. 1959. Thomas Hunt Morgan 1866-1945, A Biographical Memoir. *Biographical Memoirs*, National Academy of Sciences, Washington, DC.

Sturtevant, A. H. and G. W. Beadle. 1939. *An Introduction to Genetics*. W. B. Saunders Co. Philadelphia and London.

Sturtevant, A. H. and G. W. Beadle. 1962 (reprint of 1939). Preface, pp. 9-10, *In, An Introduction to Genetics*; "Slightly corrected reprint of 1939 edition. New 1962 Preface by Drs. Sturtevant and Beadle. 1 color plate." Dover Publications, Inc. New York, NY.

Synapsis Club Record Book 1925-1926, Division of Rare and Manuscript Collections, Cornell University Library.

Tønnesson, Ø. 1999. Sources of the History of the Nobel Peace Prize: [The Archive of the Norwegian Nobel Committee; "After 50 years the Director of the Norwegian Nobel Institute may give access to these archival sources, primarily for the purpose of historical research."] 1 December 1999. https://www.nobelprize.org/prizes/themes/sources-of-the-history-of-the-nobel-peace-prize/

Wallace, B. 1992. *The Search for the Gene*. Cornell University Press. Ithaca, New York.

Watson, J. and F. Crick. 1953. A structure for desoxyribose nucleic acids. *Nature* 171(No. 4356, April 25): 737–738.

The Anatomical Record. 1927. Program and Abstracts for the twenty-fifth annual meeting of the America Society of Zoologists, Nashville, Tennessee, December 28-30, 1927. *The Anatomical Record* 37(No. 2, Dec.): Abstracts, pp. 117-186.

The Anatomical Record. 1928. Program and Abstracts for the Joint Genetics Sections of the American Society of Zoologist and The Botanical Society of America. Friday Morning Session December 28th 1928. *The Anatomical Record* 41(No. 1, Dec. 25): Titles, pp. 107-116, Abstracts, pp. 93-110.

The Fifth International Genetics Congress. 1927. *Journal of Heredity* 18(No. 5, 1 May): 210–212. [Announcement of Muller's presentation is listed for Friday, Sept. 16; date apparently changed later – see Anonymous 1927a].

The Rockefeller Foundation. 1972. *The Rockefeller Foundation, Directory of Fellowships and Scholarships, 1917-1970*. Rockefeller Foundation, New York 1972, 412 pp.