

Drosophila Melanogaster, an Emerging Animal Model for the Study of Human Cardiac Diseases

Drosophila melanogaster, un modelo animal emergente en el estudio de enfermedades cardíacas humanas

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ABSTRACT

Background: The need to work with model organisms in medical research has revealed the usefulness of the fruit fly *Drosophila melanogaster*, considering its advantages to perform classic genetic studies and modern techniques of genome edition. Several human genes are similar to those of the fruit fly. We have developed for the first time in the country a cardiovascular line of research to study the genetics of aging, addictions and chronic consumption of substances in humans like caffeine.

Objective: The aim of this study was to provide experimental evidence that validates *Drosophila melanogaster* as a model to study human cardiomyopathies related to the pharmacological action of caffeine on the heart.

Methods: Cardiac function and the effect of caffeine were studied in semi-intact preparations of *Drosophila melanogaster*. Heart rate and the intracellular calcium transient were recorded and analyzed in 3, 7 and 40-day-old adult flies harboring one genetically encoded reporter system. Hearts of adult flies were dissected to show the myofibrillar structural organization and specific proteins such as SERCA.

Results: Aging and caffeine alter contraction rate and intracellular calcium handling in the adult heart of *Drosophila melanogaster* in a similar way as mammals

Conclusion: The study supports the use of this model of fast and easy reproductive cycle to identify the genes involved in the mechanisms through which aging, caffeine (and other substances) and environmental factors affect the heart.

Key words: *Drosophila melanogaster* - Transgenesis - Calcium - Caffeine

RESUMEN

Introducción: La necesidad de trabajar con modelos de organismos en la investigación sobre salud ha revelado las utilidades de la mosca de la fruta *Drosophila melanogaster* considerando sus ventajas para realizar genética clásica y modernas técnicas de edición del genoma. Muchos genes humanos son homólogos a los genes de la mosca. Hemos desarrollado por primera vez en el país una línea de investigación cardiovascular para estudiar la genética del envejecimiento, las adicciones y sustancias de consumo crónico en el humano como la cafeína.

Objetivo: Aportar evidencia experimental que valida el modelo de *Drosophila melanogaster* para el estudio de miocardiopatías humanas en relación con la acción farmacológica de la cafeína sobre el corazón.

Material y métodos: Se analizaron la función cardíaca y el efecto de la cafeína en preparados semiintactos de *Drosophila melanogaster*. Se registró la frecuencia cardíaca y se analizó el transitorio de calcio intracelular en moscas adultas de 3, 7 y 40 días mediante un reportero codificado genéticamente. Corazones de moscas adultas se disecaron para mostrar la organización estructural de las miofibrillas y proteínas específicas como la SERCA.

Resultados: La cafeína y el envejecimiento afectan la frecuencia de contracción y el manejo de calcio intracelular en el corazón adulto de *Drosophila melanogaster* en forma similar a lo que ocurre en mamíferos.

Conclusión: El estudio abre la posibilidad de usar este modelo de fácil y rápida reproducción en busca de genes que permitan conocer los mecanismos por los cuales el envejecimiento, la cafeína (u otros compuestos) y factores ambientales actúan sobre el corazón.

Palabras clave: *Drosophila melanogaster* - Transgénesis - Calcio - Cafeína

Abbreviations

ECC Excitation-contraction coupling

Ca²⁺_i Intracellular cytosolic calcium

SR Sarcoplasmic reticulum

SERCA Sarco/endoplasmic reticulum Ca²⁺-ATPase (Ca²⁺ ATPase pump)

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INTRODUCTION

Drosophila melanogaster as a model for the study of human diseases

Animal models for basic research studies in the health area have traditionally included mice, rats, cats, dogs and larger mammals as sheep, pigs and primates. Some of them have been ruled out due to ethical considerations and regulations regarding the care and use of laboratory animals. The fruit fly, *Drosophila melanogaster* is an organism that has become the icon of genetic studies through the works of Thomas Morgan, who provided evidence for the chromosomal theory of inheritance, genetic linkage and chromosomal crossing-over. (1)

Drosophila melanogaster is a cosmopolitan insect, with a life cycle of approximately 10 days at 25°C, which includes four phases: egg, larva, pupa and adult, and a mean adult survival rate of 70 days at 25°C. It has several advantages over other laboratory animals, as its short life cycle, its easy breeding and manipulation in the laboratory and the knowledge of its genome structure. Transgenic techniques have enabled the development of countless lines of study with overexpressed, modified, silent or deleted genes. The availability of transgenic flies from public reservoirs in Austria, Japan and the United States allow researchers from all over the world to work with these genetically modified organisms at a much lower cost than that of transgenic mice.

The *Drosophila melanogaster* genome, transcriptome and proteome have been studied and characterized at different stages of its life cycle. (2, 3) Due to the high conservation of its genes in mammals, it has become a model to study diseases such as diabetes, (4) cancer, (5) Alzheimer, (6) Parkinson, (7) obesity, (8) cardiovascular diseases, (9) and different types of human addictions in the fly. (10)

Cardiac anatomy and physiology of *Drosophila melanogaster*

The heart of *Drosophila melanogaster* is a longitudinal tube extended in the middle region from the first to the sixth abdominal segment close to the dorsal body wall. It consists of four cardiac chambers arranged in series. The pacemakers are located in the first conical chamber (first chamber) and the last chamber. (11, 12) The proteins participating in cardiac excitation-contraction coupling (ECC) are coded by similar genes in humans and the fruit fly. (13) Excitation-contraction coupling is a process that induces the increase in intracellular cytosolic calcium (Ca^{2+}_i) necessary for cardiac contraction. Then, part of this Ca^{2+}_i is released outside the cell and another part is stored in the sarcoplasmic reticulum (SR) through the ATPase Ca^{2+} pump (SERCA). The increase and decrease of Ca^{2+}_i is called Ca^{2+} transient. (14) In mammals, abnormal ECC may lead, for example, to Ca^{2+}_i overload, which is one of the main contributors to cardiac damage associated with ischemia/reperfusion (15) and to arrhythmia generation. (16)

In Argentina, our research group has been the first to use *Drosophila melanogaster* to analyze the molecular and genetic mechanisms regulating cardiac function in the adult fly heart. Studies from our laboratory have shown that aging modifies the Ca^{2+} transient in the fly similarly to what occurs in mammals, particularly in humans. (17) In addition, the laboratory has developed a fly model addicted to tobacco to study the genetic mechanisms responsible for the cardiac pathophysiology resulting from smoking.

The purpose of this work is to present experimental results showing that the heart of *Drosophila melanogaster* reproduces the results obtained in other commonly used laboratory species and even humans. This demonstration represents only an example that validates the use and importance of *Drosophila melanogaster* as a flexible model of genetic manipulation and genome editing, which in the future will allow: 1) to analyze the effect of cardiac and non-cardiac gene deregulation on heart function, 2) to apply, as in mammals, pharmacological strategies to block or exacerbate protein functions managing Ca^{2+}_i in cardiac tissue and 3) to study the molecular and genetic mechanisms of the deleterious effects of addictions.

METHODS

Flies stocks and genetic crosses

The groups of flies were amplified and kept in vials at 28 °C, partially filled with a mixture of cornmeal, glucose, agar and yeast, supplemented with 10% antifungal agent to avoid contamination.

Wild-type flies of the Canton-S strain were crossed with a homozygous line of transgenic flies expressing a genetically modified reporter system called GCaMP3 that senses increments in Ca^{2+}_i . (13). The offspring (F1) harbor a copy of the reporter system that codifies a green fluorescent protein only expressed in the heart, under one a specific cardiac promoter.

Semi-intact preparation and cardiac function analysis

The experiments with hearts of 3, 7 and 40-day-old adult flies were performed as previously described. (17) Dissections were carried out in a XTD 217 Schonfeld Optik stereoscopic microscope. Flies were briefly anesthetized with carbon dioxide (CO₂), placed on a Petri dish and fixed dorsal side down. The preparation was maintained in oxygenated, artificial hemolymph solution containing 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8 mM MgCl₂, 1 mM NaH₂PO₄, 4 mM NaHCO₃, 10 mM sucrose, 5 mM trehalose, and 5 mM HEPES (pH 7.4). Head, thorax and abdominal organs were removed. The heart was exposed, attached to the dorsal cuticle by a network of alary muscles.

During the time of fluorescent signal acquisition in the confocal microscope, a dose of 10 mM caffeine, equivalent to 2 mg/ml (final concentration) was applied 20 seconds after initiating the recording. The preparation remained incubated during 40 seconds until the end of each image acquisition. Images were analyzed using LabChart (AD Instruments, CO, USA) software.

Immunohistochemistry

Semi-intact preparations of adult flies were obtained.

Hemolymph was replaced by relaxing tamponade solution (10 mM EGTA in hemolymph) to stop the hearts, (18) which were fixed with 4% PFA for 15 minutes. They were washed three times with 3% bovine seroalbumin (SAB) dissolved in phosphate buffered saline (PBS). Then, the hearts were permeabilized with triton 0.5% in PBS during 20 minutes. They were washed again and incubated with anti-SERCA primary antibody 1:1000 (provided by Dr. Sanyal, USA). After three washings, the hearts were incubated with secondary anti-mouse antibody conjugated to Cy2 (Abcam Inc). Phalloidin 1:1000 conjugated to Alexa Fluo 594 (Life Tech) during 90 minutes and the DNA-intercalating dye, Nuclear Mask stain, were added during 15 minutes, in the dark. After a new washing cycle, hearts were mounted for subsequent visualization in an Olympus FV1000 confocal microscope.

Statistical analysis

Three, 7 and 40-day-old flies were compared using one-way ANOVA with Bonferroni correction. Conditions without and with caffeine were compared using Student's t test. Significant differences were considered for $p \leq 0.05$.

RESULTS

The cardiac muscle of *Drosophila melanogaster* is similar to the mammalian cardiac tissue

Figure 1 shows a semi-intact preparation and the characteristics of cardiac tissue in the adult fly. Typical striations of the contractile fiber arrangement are similar to those observed in the mammalian cardiac muscle. Different chambers connected by valves can be identified. At the side of the cardiac tube there are pericardial cells and extensions of alary muscles that support the heart (not shown). SERCA is detected in the zone corresponding to the intercalated disks of the adult tissue. This protein is important in functional studies, as it is responsible for Ca^{2+} reuptake into the SR.

Aging affects cardiac function and the Ca^{2+} transient

Spontaneous heart rate and Ca^{2+}_i management in

adult flies at different ages were measured. The methods used are similar to those implemented in mammalian models, but instead of using fluorescent indicators to measure Ca^{2+}_i , a genetically codified reporter system was used. (13) Figure 2A shows typical recordings of the Ca^{2+} transient corresponding to 3, 7 and 40-day-old individuals. Decreased heart rate and prolonged relaxation (assessed by the tau constant) was observed during aging. Figure 2B shows mean data.

Caffeine modifies Ca^{2+}_i dynamics in *Drosophila melanogaster*

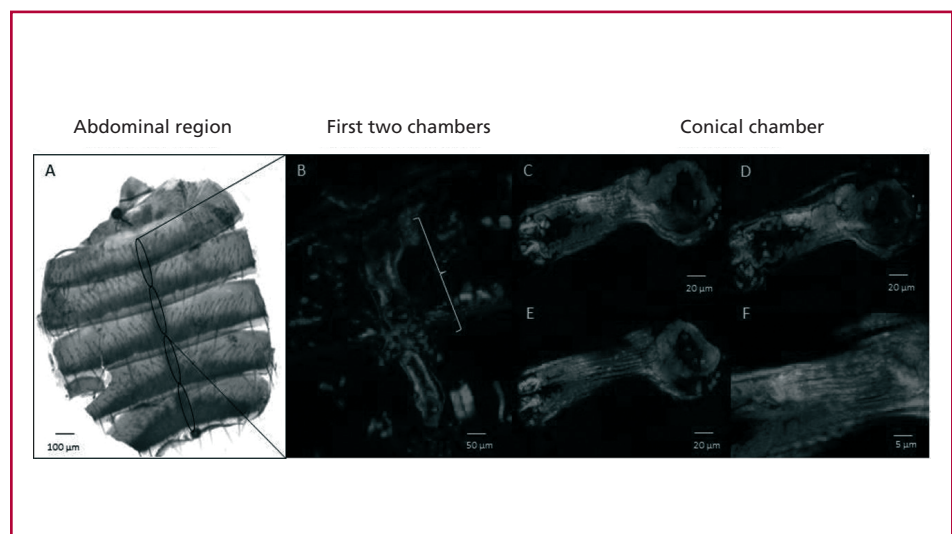
A caffeine pulse was applied in the semi-intact preparation of the adult fly heart to estimate SR Ca^{2+} content, reproducing the technique classically used in mammals. In addition, we present results describing the behavior of the cardiac muscle as a result of sustained caffeine incubation, which has not been fully studied in mammals.

Figure 3A shows a typical recording in a 3-day-old adult fly showing Ca^{2+} transients, a caffeine pulse and the subsequent transients in the presence of caffeine. Sustained caffeine administration in adult flies during 40 seconds increased the Ca^{2+} transient at all ages analyzed (Figure 3B). Ca^{2+} transient relaxation, assessed by the tau constant, was prolonged in the presence of caffeine, such effect was less evident at advanced ages, as aging prolongs the relaxation time in older adult flies (Figure 3C).

Caffeine reduces heart rate in *Drosophila melanogaster*

In our model, 2 mg/ml caffeine reduced heart rate at all ages studied (Figure 4A). Moreover, heart rate variability, called arrhythmicity index, estimated as the standard deviation of cardiac cycle duration relative to its mean value was measured. Caffeine increased the arrhythmia index only in 3-day-old adult flies, while it induced no heart rate variability at more advanced ages (Figure 4B). Also, the number of asystolic events, i.e. period in which there was absence of heart

Fig. 1. The cardiac tissue of the fly heart has a myofibrillar pattern similar to that of mammals and conserved Ca^{2+} management proteins. **A.** Transmitted light shows the abdominal region of *Drosophila melanogaster* at 10x magnification and the enhanced heart image in the dorsal medial area at the center of the abdomen. **B.** Fluorescent image of the conical chamber and the second chamber showing the myofibrillar organization in red and the cell nuclei in blue. **C-E.** Optical section of the first chamber at three different levels of depth showing fiber arrangement. **F.** **C-E** amplification at the basal region of the conical chamber to show the SERCA protein, in green, located in intercalated zones between the myofibrils. See color image in the web.



beats, was recorded. The percentage of 3 and 7-day-old young flies presenting asystolic events in the presence of caffeine was lower compared with older ages (Figure 4C). This indicates that age has influence on the effect of caffeine in the regulation of heart rate.

DISCUSSION

The development of integral research through molecular, genetic and physiological studies in model organisms to understand pathophysiological conditions in humans is a requisite for any later clinical approach. *Drosophila melanogaster* offers clear advantages over any other animal model in terms of laboratory management and generation of transgenic organisms. Even considering the evolutionary distance with humans, the study of conserved genes allows us to understand the genetic the genetic basis of cardiac injuries in humans, using experimental strategies that are difficult to successfully perform in mammals. The independence of the circulatory system from the respiratory system of the fly is beneficial for the study of genes associated with cardiac organ genesis and the specific signaling pathways of this system, without affecting the life of the individual. (11)

Previous studies from other groups investigating *Drosophila melanogaster* have revealed its cardiac ultrastructure in larvae and adults. This muscle consists of a layer of cardiac myocytes containing structural proteins as sarcoglycans, dystrophins, myosins and troponins. (18-20) In the present study, we show histological details of the adult *Drosophila melanogaster* heart, with special emphasis in SERCA, a conserved protein already observed in fly larvae, (21) which is essential for Ca^{2+}_i management in ECC. The study also demonstrates that the *Drosophila melanogaster* heart

reproduces the Ca^{2+}_i dynamics observed in mammals in different experimental situations, validating the use of the model for future genomic, transcriptomic and proteomic research to study genes and its products exposed to different agents.

In humans, aging occurs in association with cardiac function disorders such as heart failure, arrhythmias and hypertrophy, among others. (22) It is a genetically programmed process affected by the environment. The fly genes involved in cardiac senescence are homologous to those in humans. (23, 24) Impaired Ca^{2+}_i management, prolonged relaxation and arrhythmia generation are among the subcellular mechanisms responsible for cardiac function injury. (25, 26) These effects have been reproduced in *Drosophila melanogaster* (17) and some genetic characteristics of the response to aging have been identified, which in the fly also occurs with higher prevalence of fibrillation and arrhythmias. (27, 28)

In addition, caffeine is a substance whose impact on the heart has been studied in mammals, given its importance as a substance of massive consumption in the human population. One hundred milligrams of caffeine intake (a cup of coffee) in humans reaches 0.5 to 3 $\mu\text{g/ml}$ concentration in blood without mobilization of cardiac intracellular Ca^{2+} reservoirs. (29)

The effect of caffeine on heart rate depends on the dose used in animal models and studies on coffee consumption in patients. (30) Acute studies in murine embryos show that low caffeine concentrations (0.04 mg/ml, approximately 200 μM) induce increase of heart rate, whereas higher doses reduce it. (31) In this study, a dose of 2 mg/ml corresponding to 10 mM, reduced heart rate in accordance with studies performed in other experimental models with elevated

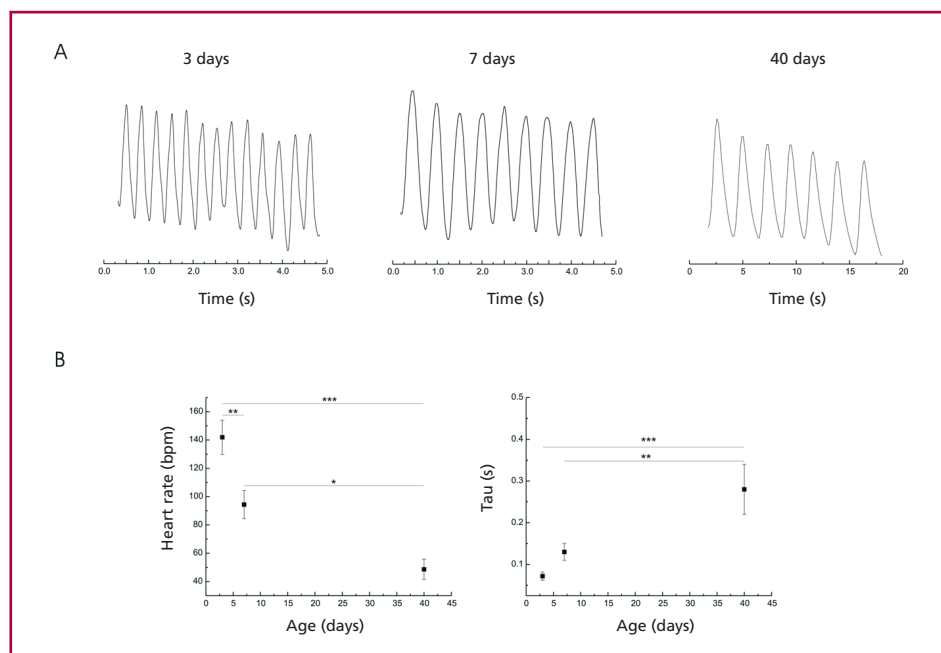


Fig. 2. Aging reduces heart rate and prolongs the relaxation time in *Drosophila melanogaster*. **A.** Typical spontaneous heart rate recordings in 3, 7 and 40-day-old flies. Heart rate is higher in 3 and 7-day-old flies than in 40-day-old flies. The time scale to represent the Ca^{2+} transients in 40-day-old flies is larger due to the significant reduction in the number of beats. Prolonged relaxation (assessed by the tau constant) is observed with age. **B.** Mean values for both parameters at each age analyzed. For heart rate: 3 days vs. 7 days ** $p < 0.01$; 3 days vs. 40 days *** $p < 0.001$; 7 days vs. 40 days * $p < 0.05$. For the tau relaxation constant: 3 days vs. 40 days *** $p < 0.001$; 7 days vs. 40 days ** $p < 0.01$.

Fig. 3. Caffeine increases the Ca²⁺ transient amplitude and prolongs the relaxation time. **A.** Recording shows the succession of spontaneous beats. The addition of 2 mg/ml caffeine increases the Ca²⁺ amplitude. Sustained incubation with caffeine modifies heart rate and relaxation. **B.** Mean data showing that incubation of 3 and 7-day-old flies with 2 mg/ml caffeine (10 mM) significantly increased the amplitude of the Ca²⁺ transient and that this effect was lower in 40-day-old flies. # p<0.05 for 3 and 7-day-old flies without and with caffeine. Caffeine effects on the relaxation constant showed an increase of tau at all ages, with significant differences in flies of 3 and 7 days old.

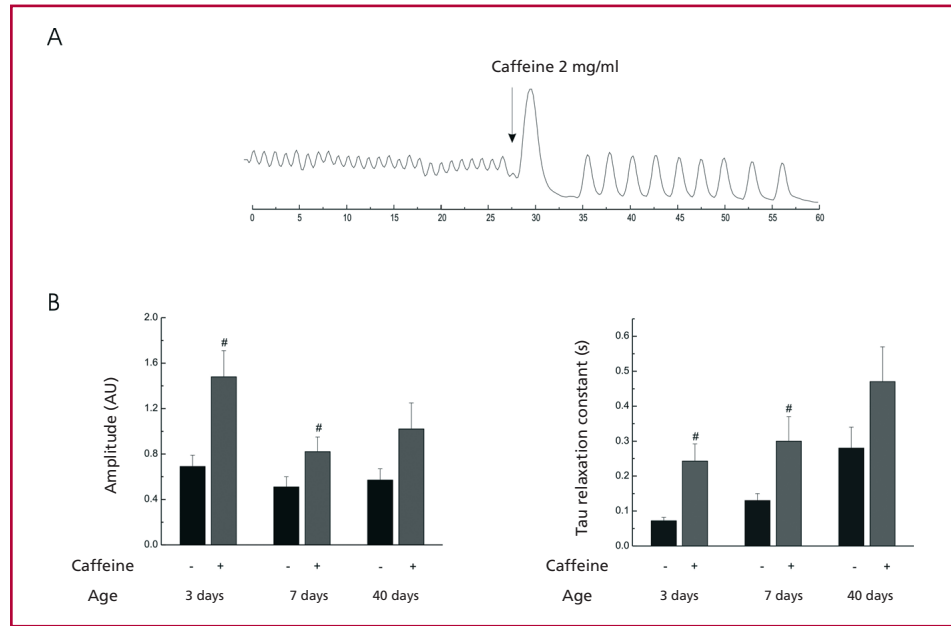
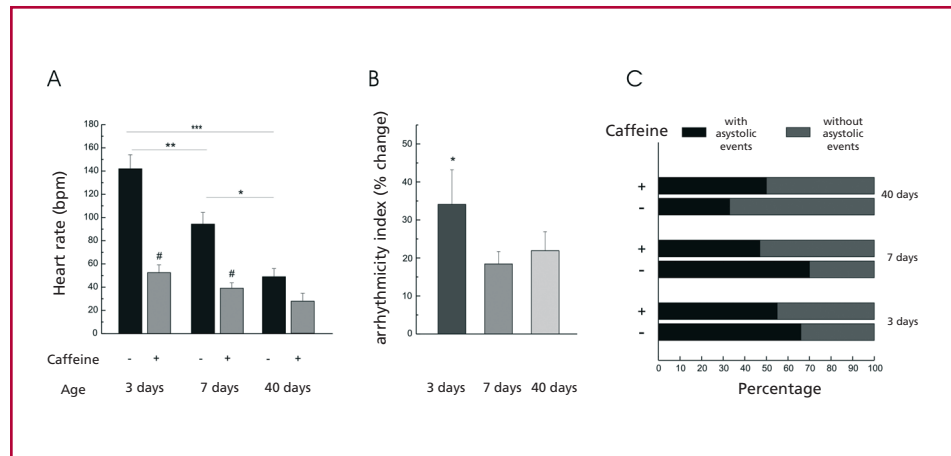


Fig. 4. Caffeine reduces heart rate and increases the arrhythmicity index. **A.** Caffeine reduced spontaneous heart rate of adult flies at all ages studied with significant differences at 3 and 7 days of age, # p<0.05. **B.** In the presence of caffeine, heart rate variability, expressed as arrhythmicity index, was significantly increased at 3 days of age. * p<0.05. **C.** The percentage of flies showing some asystolic events (absence of heart beat) was reduced in presence of caffeine, except at advanced ages (40 days), where a greater percentage of flies showed one or more asystolic events.



doses. (32, 33)

Conversely, it has been described that caffeine induces arrhythmogenesis. (30) In this work we observed that caffeine induces greater heart rate variability but reduces asystolic events, since a low proportion of flies presented some asystolic events in the presence of caffeine.

At the subcellular level, the application of a caffeine pulse in mammalian cardiomyocytes allows the estimation of SR Ca²⁺ content, as it triggers opening of SR ryanodine receptor (RyR2) channels and blocks Ca²⁺ reuptake into that reservoir. (34) This technique has been reproduced in flies but has not been fully analyzed. (13) Data provided by the present work show that, in accordance with previous studies in mammals, (35) the incubation of adult flies with caffeine prolonged the relaxation time, probably due to the inhibitory effect on Ca²⁺ reuptake into the SR through SERCA. Moreover, it increased the Ca²⁺ transient amplitude. In the mammalian model, caffeine modi-

fies RyR2 cytosolic and luminal Ca²⁺ sensitivity, (36) promoting Ca²⁺ release triggering arrhythmias. In our model, we did not study myofilament Ca²⁺ sensitivity, but we observed that, similarly to mammals, caffeine empties Ca²⁺ reservoirs through RyR2 channel opening. There are transgenic lines -conditional SERCA and RyR2 mutants, both genes conserved in humans- that could be studied to characterize other subcellular events in the presence of caffeine.

In conclusion, the homology of certain genes as the ones mentioned in this study, the response to pharmacological stimuli and genetic manipulation in *Drosophila melanogaster* supports its use as a tool for the study of the epigenetic basis of cardiac pathophysiology in humans.

CONCLUSION

The study opens the possibility of using this model of easy and quick reproductive cycle to identify the genes involved in the mechanisms through which aging, caf-

feine (or other substances) and environmental factors affect the heart.

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Conflicts of interest

None declared. (See authors' conflicts of interest forms in the website/Supplementary material).

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Supplementary material

You can find the video "Characteristics of *Drosophila Melanogaster* II" at: <https://youtu.be/lKkos7SLmgw>