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Population Effects of Calcium Phosphate Nanoparticles in *Drosophila melanogaster*: The Effects of Phase Composition, Crystallinity, and the Pathway of Formation

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Abstract

Unpredictable biological response due to the finest nanostructural variations is one of the hallmarks of nanoparticles. Because of this erratic behavior of nanoparticles in living systems, thorough analyses of biosafety must precede the analyses of the pharmacotherapeutic efficacy and simple animal models are ideal for such purposes. *Drosophila melanogaster*, the common fruit fly, is an animal model capable of giving a fast, high-throughput response as to the safety and efficacy of drug delivery carriers and other pharmacological agents, while minimizing the suffering imposed onto animals in more complex in vivo models. Here we studied the effects on the viability and fertility of *D. melanogaster* due to variations in phase composition, crystallinity, and the pathway of formation of four different calcium phosphate (CP) nanopowders consumed orally. To minimize the effect of other nanostructural variables, CP nanopowders were made to possess highly similar particle sizes and morphologies. The composition of CP affected the fecundity of flies, but so did crystallinity and the pathway of formation. Both the total number of eclosed viable flies and pupae in populations challenged with hydroxyapatite (HAP) greatly exceeded those in control populations. Viability was adversely affected by the only pyrophosphate tested (CPP) and by the metastable and the most active of all CP nanopowders analyzed: the amorphous CP (ACP). The pupation peak was delayed and the viable fly to-pupa ratio increased in all the CP-challenged populations. F₁ CPP population, whose viability was most adversely affected by the CP consumption, when crossed, produced the largest number of F₂ progeny under regular conditions, possibly pointing to stress as a positive evolutionary drive. The positive effect of HAP on fertility of fruit flies may be due to its slow absorption and the activation of calmodulin during the transit of oocytes through the reproductive tract of fertilized females. Exerted in the prepupation stage, the effect of CP is thus traceable beyond the instar larval stage and to the oogenesis stage of the *Drosophila* lifecycle.

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Notes

The authors declare no competing financial interest.

Graphical Abstract

Keywords

animal model; biocompatibility; calcium phosphate; *Drosophila melanogaster*; fecundity; hydroxyapatite; nanoparticle; orthophosphate; pyrophosphate

INTRODUCTION

Being the mineral component of all hard tissues in the body, calcium phosphates (CPs) have been the most natural choice for the inorganic component of materials for hard tissue regeneration. However, as of recently, a number of interesting properties exhibited by CPs has spurred an intense investigation into their potential use as drug delivery carriers. These properties include: (a) biocompatibility, (b) bioresorbability, (c) bioactivity, (d) nonimmunogenicity, (e) ability to capture large concentrations of therapeutic organics through adsorption because of the alternation of highly charged ionic species on their particle surface, (f) the ability to swiftly dissolve in the late acidic endosome and allow for the escape of drugs into the cytoplasm following cellular uptake before the drug is proteolytically degraded in the late lysosome, and (g) self-setting behavior and controllable viscosity in the colloidal form, among others.¹

Recently, we showed that the chemical composition of CPs can be tuned to desired degradation and drug release profiles.² The particles were also able to elicit a pronouncedly positive effect on fibroblastic, osteoblastic, and osteoclastic cells in vitro, both at the phenotypic and genotypic levels.^{3,4} Simultaneously, the structure of CP nanoparticles can be tuned to elicit a finite antibacterial response even in the absence of the delivered antibiotics.^{5–7} All these findings have pointed in the direction of extraordinary properties latent in a very common material, i.e., the bone biomineral. Because it is an extremely low-cost material, readily abundant in nature, optimization of its properties for intense and selective (healthy vs pathogenic cells) therapeutic effects has the potential to grow into a means for bridging the gap between the extraordinary medical technologies reported in the literature and the comparative scarcity of the very same technologies available on the market.⁸ However, although the effects of CP nanoparticles have been widely examined in vitro, i.e., in static 2D cell culture models, they cannot be directly correlated to their effects on living

organisms during their entire lifecycles, necessitating the use of appropriate in vivo models. In this study, we extend the effort to understand the pharmacotherapeutic potential of CPs by analyzing the biocompatibility and the reproductive and antibiotic effects of orally delivered CPs of different phase compositions, using *Drosophila melanogaster* as the animal model. *Drosophila*, a.k.a. common or vinegar fruit fly, is a simple and the most commonly used nonmammalian animal model.^{9–11} It is capable of giving a fast and high-throughput response as to the cytotoxicity and the therapeutic efficacy of drug delivery carriers and other pharmacological agents,^{12,13} while minimizing the suffering elicited to larger, CNS-containing animal counterparts in more complex in vivo models.

The different CP nanopowders analyzed here differed fundamentally in terms of their chemical composition, crystallinity and mechanism of formation. The rationale for testing these variations was to cover a broad spectrum of properties and elucidate the effects on each in an in vivo model. The assessment of the biological effects of nanoparticles in animal models has been usually limited to variables such as particle size and shape, the caveat of which is their multivariable nature, e.g. size of spherical particles cannot be modified without affecting their surface curvature and thus shape, whereas their shape cannot be modified without affecting the geometric aspect ratio and, thus, the effective size. In this study we go beyond these classical variables while focusing on parameters of interest for the biomineralization and in vivo remodeling of CPs. The latter are complex, multiphase and multistage processes that involve transitions between different chemistries, crystallinities and pathways of formation of CPs. Specifically, the analyzed CP nanopowders included hydroxyapatites precipitated under alkaline (HAP1) or acidic (HAP2) conditions, calcium pyrophosphate (CPP), the only CP phase that is not an orthophosphate, and amorphous CP (ACP). We observed the viability effects of their ingestion on the healthy populations of *D. melanogaster* across two generations of progenies, as a prelude to their later study as drug delivery carriers for treating specific pathologies in the same animal model. *D. melanogaster* has been used before as the animal model for oral drug delivery, nanoparticle biocompatibility and organ distribution¹⁴ studies, but to the best of our knowledge, this is the first study looking at the effects of its interaction with CP nanoparticles.

MATERIALS AND METHODS

Synthesis and Characterization of Different CP Nanopowders

Four CP powders that differed in phase composition, crystallinity or mechanism of formation were synthesized and compared in this study. Two of them were hydroxyapatite (HAP) powders, one of which was precipitated under alkaline conditions ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, HAP1) and the other one of which was precipitated under acidic conditions ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, HAP2) and transitioned from dicalcium phosphate anhydrous, a.k.a. monetite, to HAP during overnight heating at 80 °C. β -calcium pyrophosphate ($\beta\text{-Ca}_2\text{P}_2\text{O}_7$, CPP) was synthesized as the only pyrophosphate CP phase, whereas amorphous calcium phosphate (ACP) was the only noncrystalline CP. The synthesis of different CP powders followed previously established protocols and involved precipitation from aqueous solutions, alongside annealing at 800 °C for 2 h in air for CPP. Briefly, to make HAP1, 400 mL of 0.06 M aqueous solution of $\text{NH}_4\text{H}_2\text{PO}_4$ (Fisher Scientific) containing 25 mL of 28% NH_4OH

was added dropwise to the same volume of 0.1 M aqueous solution of $\text{Ca}(\text{NO}_3)_2$ (Fisher Scientific) containing 50 mL of 28% NH_4OH (Sigma-Aldrich), vigorously stirred with a magnetic bar (400 rpm) and kept on a plate heated to 50 °C. After the addition of $\text{NH}_4\text{H}_2\text{PO}_4$ was complete, the suspension was brought to boiling, then immediately removed from the heater and air cooled to room temperature. Stirring was suspended and the precipitate together with its parent solution, the final pH of which was 10.6, were left to age in atmospheric conditions for 24 h. After the given time, the precipitate was washed once with deionized (DI) H_2O , centrifuged (10 s at 3500 rpm), and let dry overnight in a vacuum oven (Accu Temp-19, Across International) ($p = -20$ mmHg) at 80 °C. HAP2 was prepared by mixing 400 mL 0.33 M $\text{Ca}(\text{NO}_3)_2$ and 400 mL 0.25 M $\text{NH}_4\text{H}_2\text{PO}_4$ containing 10 mL 28% NH_4OH , with all the other conditions being the same as for the synthesis of HAP1. The pH of the supernatant following the precipitation reaction was 5.2. ACP was made by abruptly adding a solution containing 100 mL 0.5 M $\text{Ca}(\text{NO}_3)_2$ and 7 mL 28% NH_4OH into a solution comprising 100 mL 0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$ and 4 mL 28% NH_4OH . The fine precipitate formed upon mixing was aged for 15 s, before it was collected, centrifuged, washed with 0.14 w/v% NH_4OH , centrifuged again, washed with ethanol and dried overnight at low pressure ($p = -20$ mmHg) and room temperature, and stored at 4 °C to prevent spontaneous transformation to HAP. The pH of the supernatant following the precipitation reaction was 9.3. To synthesize CPP, we added 0.2 M $\text{Ca}(\text{NO}_3)_2$ solution containing 3 mL of 28% NH_4OH dropwise to 0.25 M $\text{NH}_4\text{H}_2\text{PO}_4$ supplemented with 28% NH_4OH until pH 6.8 was reached. Following precipitation and drying, the loose powder was annealed at 800 °C for 2 h (Across International), with the heating and cooling rates of 10 °C/min.

The morphology of the CP nanopowders was analyzed by means of a Hitachi S-4300SE/N scanning electron microscope (SEM) at the Lawrence Berkeley National Lab, using the electron beam energy of 15 kV. High-resolution transmission electron microscopy (HR-TEM) analysis was carried out at the National Center for Electron Microscopy, on a FEI monochromated F20 UT Tecnai HR-TEM under the electron acceleration voltage of 200 kV. The phase composition of CP particles was confirmed on a Bruker D2 Phaser X-ray diffractometer. Interplanar distances (d) with strongest reflections on X-ray diffractograms were correlated with Miller indices (hkl), the diffraction angle (θ) and lattice parameters for the hexagonal lattice of HAP ($P6_3/m$ space group; $a = 0.937$ nm and $c = 0.688$ nm) using the following equations:

$$\lambda = 2d_{hkl} \sin \theta_{hkl} \quad (1)$$

$$1/d_{hkl}^2 = 4/3((h^2 + hk + l^2)/a^2) + l^2/c^2 \quad (2)$$

The average crystallite diameter (r) was estimated by applying Debye–Scherrer's equation on the half-widths of (002) ($2\theta = 25.90$ deg) and (217) ($2\theta = 31.30^\circ$) diffraction peaks of

HAP and CPP, respectively, expressed in radians ($\beta_{1/2}$), the diffraction angle (θ), and using 1.5418 Å as the wavelength of $\text{CuK}\alpha$ as the radiation source (λ):

$$r = 0.94\lambda/\beta_{1/2}\cos\theta \quad (3)$$

The Oregon R strain of *Drosophila melanogaster* (Carolina Biologicals) was reared under regular light-dark cycles at variable room temperature and fed the standard medium purchased from the lab of R. Dubreuil, University of Illinois, Chicago. To make nanoparticle-infused fly food, we heated and liquefied the solid medium before nanoparticles were added to the liquid food solution at 3 mg of nanoparticles per gram of food. The nanopowders were first finely ground in a mortar, then mixed vigorously into the liquid food for 3–5 min. Once the nanoparticles were homogeneously mixed throughout the food, the food was aliquoted into food vials and allowed to cool. For each type of nanoparticles, 3 sets of flies were crossed, with each set consisting of 10–12 (each) of male and female flies placed into the particle-infused food. Flies were then switched to a new vial of particle-infused food every other day for a total of 10 vials per set and 30 vials total for each type of nanoparticle. The adult flies were then discarded. The vials of nanoparticle-infused food were then observed for the presence of eggs, larvae and pupae. F1 pupae and eclosed flies were then counted. To determine if ingestion of nanoparticles had any cross-generational effects, 10 F₁ single fly crosses for each nanoparticle type were performed. F1 adults were allowed to breed and lay eggs for 3 days in the standard medium without any nanoparticles. After 3 days, the adults were discarded and vials observed for the presence of eggs, larvae and pupa. Eclosed F₂ flies were then observed and counted. Numbers of pupa cases for each set of ten vials were averaged across three sets and plotted as a function of time. The number of eclosed flies (m) was calculated using eq 4, where i is the number of experimental vials ($i = 10$), j is the number of days of incubation ($j = 52$), k is the number of sets of vials ($k = 3$), and $m_{i,j,k}$ is the number of eclosed flies in vial i , on day j , and in set k :

$$m = \frac{\sum_{k=1}^3 \sum_{j=1}^{52} \sum_{i=1}^{10} m_{i,j,k}}{k} \quad (4)$$

The number of viable pupa cases forming per *Drosophila* population was estimated by subtracting the numbers of viable pupae forming on subsequent days, summing these numbers and normalizing per number of vials, as shown in eq 5, where $n_{i,j,k}$ is the number of healthy pupae observed in vial i , on day j , and in set k :

$$n = \frac{\sum_{k=1}^3 \sum_{j=1}^{52} (\sum_{i=1}^{10} n_{i,j,k} - \sum_{i=1}^{10} n_{i-1,j,k})}{k} \quad (5)$$

Drosophila crops were dissected from adult flies who had been fed the nanoparticle-infused food. Dissected crops were fixed in 4% paraformaldehyde for 15 min and washed 3 times in 1× phosphate buffered saline (PBS) for 5 min each. Tissue was then blocked in a 1× PBS

solution containing 0.1% Triton-X and 1% bovine serum albumin overnight at 4 °C. Crops were stained in the blocking solution using AlexaFluor 568 Phalloidin 1:400 (Life Technologies), OsteoImage bone mineralization assay 1:100 (Lonza) and DAPI 1:1,000 for 1 h at room temperature. After 1 h, crops were washed 3 times for 30 min each in the blocking solution, then mounted onto glass slides using ProLong Diamond antifade mountant (Life Technologies) and cured overnight in the dark. Immunofluorescent images of the crops were taken using a Zeiss710 meta confocal optical microscope in the microscopy core facility at the University of Illinois, Chicago.

RESULTS AND DISCUSSION

3.1. Physicochemical Characterization

The purpose of this study was to (a) assess the difference in the biocompatibility, viability and reproductive response to ingestion of CP nanoparticles of different phase compositions, crystallinity, and mechanism of formation; and (b) assess their potential applicability as oral drug delivery carriers to treat infected organisms. To that end, the parental generation of the common fruit fly (*D. melanogaster*), F₀, and F₁ progeny were fed a diet containing 0.3 wt % of dispersed nanoparticles of one of four different CP phases: alkaline HAP, the CP phase comprising the mineral component of mammalian bone tissues, precipitate either under alkaline (HAP1) or acidic (HAP2) conditions; CPP as the only CP that is a pyrophosphate, not an orthophosphate, containing the P₂O₇⁴⁻ group instead of the H_xPO₄^{x-3} one; and amorphous CP (ACP), a phase that is highly active, metastable, and quick to transition to HAP, being its precursor in the process of biomineralization. To ensure that any difference in response is not due to a difference in the particle size or shape, all CP phases were prepared with the goal to produce as similar size and shape as possible. Figure 1 displays the SEM images of the four different CP nanomaterials, all of which possessed an indistinguishable level of similarity in terms of the particle size and morphology. CP nanoparticles were spherical and averaged at ~100 nm in diameter for all CP phases except CPP, in which case the particles were 150–200 nm in size on average. The hexagonal crystallographic structure of HAP was confirmed by indexing the HR-TEM lattice fringes. The most prominent plane was (002), crossing the *c*-axis perpendicularly, with the characteristic interplanar spacing of 1.72 Å. Meanwhile, all the four different CP nanopowders were monophasic in composition, as seen from their respective diffractograms (Figure 2). Only the amorphous hump, present in the region of the most prominent diffraction peaks of HAP and brushite/monetite, was observed in the ACP pattern. Because of the thermal treatment, the crystallinity of CPP was higher than that of HAP1 and HAP2, which were in a similar range when measured along the *c* axis, on (002) crystallographic planes, displaying the crystallite sizes of 17.0 and 27.8 nm, respectively, per Debye–Scherrer’s equation.

3.2. Population Analysis

The reproductive effects of the ingestion of different CP phases on *D. melanogaster* were assessed by measuring daily the number and the gender of viable flies eclosing from the populations exposed to the nanoparticle treatment as proxies for female fertility and larval viability and comparing them with the control, untreated population throughout a 52-day incubation period. As seen in Figure 3a, the total viability in terms of the number of viable

flies eclosed from the two HAP-challenged populations after the 30th day of the incubation was higher than the control. Between the two types of HAP, the one made under alkaline conditions, HAP1, and directly precipitated as such proved to be more conducive to *Drosophila* fertility and/or larval viability than the one made under the acidic conditions, HAP2, and having monetite as an intermediate. Different synthesis methods can often produce nanoparticles with diametrically opposite properties,¹⁵ and we have previously used a difference in the pathway of formation of HAP in an identical synthesis method to control the release rates of adsorbed drugs.¹⁶ Therefore, these findings are not surprising. In contrast, the viability of the control population was in the same range as that of ACP- and CPP-challenged ones. Between the 30th and the final, 52nd day of the treatment, the control population began to supersede, in terms of the number of eclosed flies, those of ACP- and CPP-challenged ones. Still, HAP1-challenged population retained the highest viability of them all (Figure 3b).

The timeline of emergence of viable flies from their respective pupae shows that all populations, both the control and the CP-challenged ones, began to eclose on day 13 following the laying of the first eggs (Figure 4). However, unlike the control population, which eclosed consistently for the first week and then began to eclose in cycles, peaking on days 27, 34, 41 and 48, i.e., exactly with a week-long phase, the CP populations, with the exception of CPP, did not induce this regular and pronounced periodicity (Figure 4a). The last animal eclosed on day 27 in the ACP population, on day 31 in the HAP2 population and on day 48 in the HAP1 population, whereas only CPP and control populations produced flies until the end of the 52-day long incubation period, albeit gradually decreasing the output of the F₁ offspring. The emergence activity of all *Drosophila* populations, including both the control and the CP-challenged ones, peaked on day 20, as shown in Figure 4b. However, the peak of eclosing was significantly greater in populations challenged with the two forms of HAP than for the other ones, including the control. The total developmental time from egg to adult emergence was longer than the typical 9–11 days seen at the constant temperature of 25 °C, the reason being the higher and variable temperatures and air pressures of the ambient conditions used in the study. Namely, the study was performed in a nonair-conditioned lab during the summer days of June and July in Chicago, IL, with temperatures varying from as low as 13 °C on the zeroth day to as high as 33 °C and barometric pressures varying from 29.5 to 30.2 mmHg during the given 52-day period. Temperatures both lower and higher than the ideal 22 °C are known to delay the development of *Drosophila*.¹⁷ Additionally, unstable temperatures have been known to cause variations in the size of larvae with no deterrents to their development,¹⁸ adding to the effect of crowding,^{19,20} which reduces the body size but produces no known fertility or developmental defects. For this reason, the body size of both larvae and eclosed adults of *Drosophila*, often affected by parameters outside of the control, were not taken into consideration during the viability analysis.

Viability of the population challenged with different CP phases was also assessed by measuring daily the numbers of pupae that were yet to be eclosed, while discarding the empty pupa cases (Figure 5). Following the timeline and the extent of pupation in flies exposed to the treatment can be informative of the effects of the treatment.²¹ The control population pupated more copiously at the onset of pupation, on day 5 of the egg incubation, and only CPP-challenged population produced comparable levels of pupation. However,

from this earliest stage and throughout the entire incubation period, the tendency for ACP- and CPP-treated animals to form the least number of pupae was obvious. This trend directly corresponded to the lowest number of eclosed flies for these two fly populations in total (Figure 3b). On day 10, pupation of HAP1 population exceeded that in the control and by day 13 it tripled over the control. Thus, the number of pupae increased from 36.4% of the control on day 5, the first day pupae were observed, to 310.8% of the control on day 13. This effect was similar, albeit somewhat less pronounced for HAP2 (Figure 5). The trend of dominant pupation in HAP1 population over the control and other CP populations continued through day 24 when pupae in the control population overtook and, despite the obvious oscillations in pupation (Figure 5a), began to outnumber the other CP populations. Figure 5b demonstrates the delayed pupation peak due to the treatment with CP nanopowders. Such developmental delays were previously observed in *D. melanogaster* orally dosed with magnetite nanoparticles, although they were causative of a number of morphological defects absent in this case, and were ascribed to the dyshomeostasis of Fe along the anterior-posterior axis of the fertilized eggs.²² In spite of the delayed peaking, the pupation peaks of both HAP populations were significantly higher than those in ACP, CPP and control populations (Figure 5). Another difference between pupation in the control population and pupation in the CP-treated populations is that the former was cyclical in nature, peaking at approximately every 15 days. This cyclical peaking, corresponding to the peaking of the fly emergence from pupae (Figure 3a), was less pronounced in populations challenged with CPs.

To elucidate whether the viability reduction in ACP and CPP populations was because the flies did not eclose after they pupated or because they died somewhere along the way before the pupation stage, we compared the viable eclosed numbers vs the viable pupa numbers. As seen by comparing Figures 6a and 3a and Figures 6b and 3b, there is a direct correspondence between these two at both days 30 and 52 of the incubation time. Namely, by day 30, both HAP populations exceed in number of both pupae and eclosed flies those of the control, ACP, and CPP populations, which were approximately at the same level. By the final day of the experiment, day 52, whereas HAP1 population exceeded in both the number of pupae and eclosed flies those of the control, the rest three CP nanopowders produced the opposite effect, having lowered both the number of eclosed organisms and the extent of pupation. In fact, the order and the approximate ratios between pupa numbers in different populations directly correspond to those between the number of eclosed viable flies. Comparing the exact ratio of the numbers of eclosed viable flies to the numbers of pupae shows that in the early stages of the development, the efficiency of the transition from pupae to flies is increased for all four CP-challenged populations (Figure 6c). Comparing this ratio on the final day of the experiment shows practically identical values for the control and the two HAP populations and a preservation of the mild increase for the stressed, ACP and CPP populations (Figure 6d). When it comes to explaining the viability boost observed in HAP1 population, no significant difference in this ratio compared to the control directly indicates that HAP1 nanopowder affects the fertility, somehow increasing the health of larvae in the prepupation stage of the fly lifecycle. Similarly, the moderately adverse effects of ACP and CPP powders are not due to their causing developmental anomalies or delays, but are due to their being toxic to one or all forms of larvae, the result of which was the reduced larval

population and the correspondingly reduced number of third instars that successfully pupated when compared to the control and other particle treatments. The effects of CP nanopowders, consequently, must be exerted either at the oogenesis stage or in one of the three instar larval stages.

A consistently higher number of eclosed females than the males was observed among the viable flies in all the experimental groups, including the control, at the end of the 52 day incubation period (Figure 7). The female-to-male sex ratio (F/M) was particularly skewed in favor of females early on through the incubation period, but began to rebalance later on. Thus, for example, for the HAP1-challenged population, F/M was 2.8 after the third day of eclosing, i.e., day 15 of the total egg incubation, but dropped to 1.86 5 days later, 1.65 15 days later and reached 1.44 at the end of the 52-day incubation period (Figure 7). It was only in the CPP-challenged population that F/M was consistently around 2 and did not show the trend of decreasing over time. Still, only the two HAP populations produced statistically significant increases in F/M compared to the reference value of 1 (Figure 7). Genetic distortion causing female-biased progeny is well-documented in *Drosophila* and is linked to the meiotic drive of the two different X chromosome sequences against the Y chromosome,²³ an effect that, however, suffers from a strong fertility disadvantage.^{24,25} Like the viability effect, it is uncertain whether the distorted sex ratio effect is exerted at the germline parent level or at the larvae level. Since even the control populations exhibited the same, albeit statistically insignificant increase F/M, it is possible that temperature variations may be responsible for this effect. Increased female-to-male sex ratios in *D. melanogaster* maintained at temperatures varying in the 20–30 °C range compared to those maintained at the constant room temperature of 25 °C were observed earlier.²⁶ This temperature effect on the fruit fly gender falls under the domain of thermosensitive sex selection, which is frequently used in zoology of fish and reptiles.²⁷ Chronic cold exposure can shift the sex ratio toward a male-biased extreme,²⁸ whereas spiroplasma endosymbionts, the maternally inherited micro-organisms that infect arthropod species including *Drosophila* and act as reproductive manipulators by killing exclusively sons of infected mothers may be a factor involved in this variation.²⁹ Genotoxic effects have been observed in *Drosophila* dosed with inorganic nanoparticles,^{30–32} but they are improbable to occur with CPs, especially at the low concentration in media of 0.3 wt % used in this study. Even more elevated calcium levels would have been expected to produce a more systemic, cell signaling effect rather than a genotypic one. The general lack of mutagenic effects associated with the oral use of CP, a common raising agent in food industry, a.k.a. E341, complied with no phenotypic aberrations observed in flies subjected to a diet supplemented with CP in the nanoparticulate form.

Indeed, to test if there were any downstream germline effects of the CP consumption, single F₁ male/female crosses were set up within each CP-challenged population using ten single pairs of virgin females and males per population. They were fed regular, CP-free diet and followed for viability throughout a 31-day period. These experiments noted no negative effects on the progeny, as demonstrated by the comparatively same levels of pupation and fly eclosing observed in the control F₂ population and in HAP1, HAP2, and ACP challenged ones (Figure 8). Interestingly, the only population to have significantly exceeded that of the control in terms of the extent of pupation was the one whose parental, F₁ line was fed CPP-

containing diet. In terms of the rate of eclosing viable flies, the progeny of the CPP-challenged F₁ line significantly surpassed all the other populations, too. That the F₁ CPP population, which showed the signs of the highest stress together with ACP (Figures 3 and 6) and whose viability was adversely affected by the CP consumption, would produce the more viable F₂ progeny under regular conditions is surprising, but not unexplainable. Moderate environmental stress, imposed in the form of ingested CPP nanoparticles, may have selected for genetic variants among the population that could best tolerate the stress and thus have a reproductive advantage over the untreated control and other CP-challenged populations. *D. melanogaster* females have previously been shown to make oviposition decisions that are at mismatch with the larval nutritional response, that compromise the adult size and fertility, and that, thus, do not appear to benefit the larvae.³³ Notwithstanding the taxonomic and/or strain purity risks, a seemingly counterintuitive choice of developmental pathways for the direct, F₁ progeny made by the P₀ female, as through subjugation to environmental stress that affects the population at large but does not decimate it entirely, may appear as a positive evolutionary drive when viewed in a bigger frame, that is, further down the family tree.

These findings showing a definite effect of orally consumed CP nanopowders on the reproductive viability of *Drosophila* may be rooted in the key effect of calcium on the activation of mature oocytes during the passage of the egg through the reproductive tract of the fertilized female. The current model³⁴ states that the mechanical force exerted on the oocyte during this postfertilization transit activates mechanosensitive Ca²⁺ channels and allows the entrance of Ca²⁺ into the cytoplasm where it activates calmodulin by binding to it. Activated calmodulin acts on downstream effectors such as Ca²⁺/calmodulin-dependent kinase CaMKII and phosphatase calcineurin. While activation of CaMKII inhibits phosphorylation of Emi2, a spindle checkpoint regulator, activation of calcineurin removes the CDC20 phosphorylation, yet another spindle assembly checkpoint regulator of the cell cycle, off the anaphase promoting complex, APC/C, thus releasing the inhibition of APC/C and allowing the progression of the oocyte through the anaphase.³⁵ Therefore, increased cytosolic calcium concentrations, propagating in waves across oocytes, induce the egg activation and completion of meiosis.³⁶ *Drosophila* has no ability to consume solid matter and feeds exclusively on mushy, decaying fruit and other organic matter with varying degrees of pH. However, dispersing nanoparticles inside such food can allow for their consumption and passage from the esophagus and into the food storage organ, crop. After the dissection and immunofluorescent staining of the *Drosophila* organs, CP nanoparticles were seen exclusively in the crop (Figure 9), and were absent in other organs, suggesting the most probable degradation of the nanoparticles in the acidic milieu of the anterior midgut before biodistribution and excretion in the ionic, dissolved form. The release of the CP nanoparticles as a food component from the crop and into the anterior midgut for digestion and absorption is bound to degrade CP nanoparticles into constitutive, Ca²⁺, PO₄³⁻/P₂O₇⁴⁻ (and OH⁻ in case of HAP) ions because of the acidic milieu of the copper-cell region of the anterior midgut. Namely, although pH of the crop is little regulated and largely determined by its food content, the anterior midgut transitions from neutral to acidic (pH < 4), which is followed by the mildly alkaline posterior midgut (pH 7–9) and then by the slightly acidic hindgut (pH 5).³⁷ Calcium orthophosphates (HAP, ACP) are stable under basic and neutral

conditions, but rapidly dissolve at $\text{pH} < 3\text{--}4$, thanks to which they are used as pH-controlled, smart drug and gene carriers.³⁸ Excess protons first break down the hydroxyl channel structure running along the central, screw axis of the *P63/m* HAP hexagons and acting as a crystallographic cornerstone of its stability.³⁹ They also protonate surface phosphates, causing the temporary transition of the sparsely soluble HAP structure first into one with an octa-CP-like surface composition and then—per the Ostwald-Lussac rule—one with a brushite-like surface composition. In both of these structures (OCP and brushite), water molecules are intercalated between calcium-rich crystalline layers and partially protonated phosphates are more hydrolyzable. In brushite, in particular, (010) planes, composed of alternating rows of HPO_4^{2-} ions and $\text{Ca}^{2+}\text{-H}_2\text{O}$ clusters, both of which are oriented in the [101] direction, are fully hydrated, creating a phosphate-rich cleavage plane lying between the two nearest water layers.⁴⁰ With a further drop in the pH, this hydrolysis leads to reduced Ca/P molar ratio down to 1, the region of highly soluble mono-CP, as well as to an increased content of crystalline water, at which point the complete dissolution usually rapidly ensues. The dissolution of CP increases the blood calcium levels and may affect the oogenesis through above-mentioned Ca^{2+} -dependent effect.

These findings also indicate that different CP phases can have drastically different effects on the viability and reproductive capacity when orally delivered. The composition of CP affects the viability of flies, but so does crystallinity as well as the pathway of formation to a minor degree. The most drastic drop in viability was observed in populations treated with CPP and ACP nanopowders (Figure 3b). CPP possesses the pyrophosphate group, where two phosphates are linked by an oxygen atom, requiring the cleavage of this oxygen bridge for the group to be broken down to metabolizable, elementary phosphates. In neutral aqueous solutions, this reaction may require decades to proceed,⁴¹ but is facilitated in vivo by phagocytosis and the action of enzymes, such as alkaline phosphatase.⁴² It is conceivable that this elementary physicochemical hindrance can inhibit the absorption and affect the fertility of flies. In turn, ACP is typified by the metastable, comparatively more reactive nature than that of other CPs, which may explain the reduction in survivorship of flies ingesting it as a food additive. This coincides with the earlier demonstration of the most intense antibacterial effects by this phase, when compared against monetite, CPP and HAP.⁴³ In analogy with the faster absorption and pharmacokinetic activity of amorphous drugs compared to their crystalline counterparts,⁴⁴ it is conceivable that the overly rapid degradation and absorption of ACP may affect the viability of larvae or overall fertility due to a higher burst in calcium levels compared to the more leveraged levels of it in flies dosed with more slowly absorbed HAP. Regarding the two different forms of HAP, the one directly precipitated at a higher pH produced a more positive effect on the overall F1 population in *Drosophila* than the phase formed indirectly and at a lower pH.

CONCLUSION

A hallmark of nanoparticles is the unpredictable effect of their finest structural variations on the biological responses that they elicit.⁴⁵ Minor changes in nanoparticle size,⁴⁶ shape,⁴⁷ or surface charge⁴⁸ can cause dramatic differences in the biological response, the reason for which nanoparticles require markedly more detailed and versatile safety tests compared to their microparticulate counterparts of the same composition.⁴⁹ Because of this erratic

behavior of nanoparticles in living systems, thorough analyses of safety effects must precede the analyses of the pharmacotherapeutic efficacy. Observing increased mortality or developmental defects can be enough to put a barrier on the development of the given particles as drug delivery carriers or for other medical applications. In spite of the documented biocompatibility and excellent biosafety profiles of CPs,⁵⁰ there is always a finite possibility that CPs in the nanoparticulate form can cause a diametrically opposite effect, for which reason they must be tested for safety before they are being tested for pharmacotherapeutic efficacy. In this study we tested the effect of CP nanoparticles delivered orally on the development of *Drosophila* from the egg to the adult organism. For comparison purposes, CP nanoparticles of almost identical sizes and shapes were synthesized, differing in (a) crystallinity (amorphous vs crystalline), (b) chemical nature of the phosphate group (orthophosphate vs pyrophosphate), and (c) pathway of formation (directly formed HAP vs monetite as an intermediate). Interestingly, each of these three variables affected the viability and/or fertility of the fruit flies. The most viable *Drosophila* population, in terms of both the total number of eclosed viable flies and the pupation extent, was the one challenged with HAP. It was the only population whose viability exceeded that of the control population. Viability was adversely affected by the pyrophosphate phase, CPP, presumably because of its being more difficultly metabolizable than the orthophosphates, although the facts that CPP was the only thermally treated CP phase and that its particle size was slightly larger than that of its as-precipitated counterparts cannot be discarded with certainty from the list of potential factors affecting fertility. The viability of the flies was also negatively affected by the consumption of the amorphous CP phase, presumably because of its metastability and high activity. Still, none of the tested CP phases produced adverse effects on the cross-fertilized F₂ progeny, indicating comparatively high safety of their use as drug delivery carriers, the effects of which we will test in the next part of this investigation into the curious interaction between the two commons: the fruit fly and the bone mineral.

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Figure 1.

(a–d) Scanning and (e) high-resolution transmission electron micrographs of different monophasic CP nanopowders: (a, e) HAP1, (b) HAP2, (c) ACP, and (d) CPP. The 1 μm bar on the left corresponds to all SEM images.

Figure 2.

X-ray diffractograms of the four different monophasic CP nanopowders prepared: HAP1, HAP2, ACP, and CPP. Crystallographic planes corresponding to the given phases are labeled with the following symbols: HAP, ▲; CPP, ●.

Figure 3. Total number of viable eclosed fruit flies per set of vials ($n = 3 \times 10$) after (a) 30 and (b) 52 days of ingestion of different CP nanoparticles. Data points represent averages ($n = 3 \times 10$) and error bars represent standard deviations of the mean. * represents confidence interval, $p < 0.05$, whereas ** represents $p < 0.005$ compared to the control.

Figure 4.

Timeline of emergence of viable *Drosophila* from different egg populations. The arrow and the dashed line denote the day 20 peak of eclosing, coinciding for all the fly populations. Arrows and numbers 1–5 indicate peaks in the eclosing activity of the control population. Data points represent averages ($n = 3 \times 10$) and error bars represent standard deviations of the mean ($n = 3$).

Figure 5.

Number of healthy pupae observed in the control population and the populations challenged with different CP nanopowders on different days of exposure. The inset shows the delayed maxima of the pupa stadium in populations challenged with nanopowders compared to the control population. Data points represent averages ($n = 3 \times 10$) and error bars represent standard deviations of the mean ($n = 3$).

Figure 6.

Total number of healthy pupae forming per set of ten vials ($n = 3 \times 10$) in different CP nanoparticle-challenged populations and the control population over (a) 30 and (b) 52 days of the incubation time, along with the ratio of pupae to eclosed flies after days (c) 30 and (d) 52. Data points represent averages ($n = 3 \times 10$) and error bars represent standard deviations of the mean. * represents confidence interval, $p < 0.05$, whereas ** represents $p < 0.005$.

Figure 7.

Female-to-male sex ratio among viable flies eclosed from different CP nanoparticle-challenged populations and the control population by different time points in the incubation. Data points represent averages ($n = 3 \times 10$) and error bars represent standard deviations. Dashed line represents the female-to-male ratio of 1. * represents the significant statistical difference with the confidence interval $p < 0.05$, whereas ** represents the extremely significant statistical difference with the confidence interval $p < 0.005$.

Figure 8.

Total numbers of (a) viable pupae and (b) the average number of viable eclosed flies forming per set of ten vials in different F_2 cross-populations subjected to a regular diet over 31 days. Data points represent averages (a, $n = 3 \times 10$; b, $n = 10$) and error bars represent standard deviations of the mean.

Figure 9.
(a) Control *Drosophila* crop, and (b) *Drosophila* crop containing HAP nanoparticle agglomerates (red). Cytoskeletal actin fibers are stained in blue and cell nuclei in green.