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Molecules and Cells

Dietary Hempseed Meal Intake Increases Body Growth and Shortens the Larval Stage via the Upregulation of Cell Growth and Sterol Levels in *Drosophila melanogaster*

Min Jung Lee[1,5], Min Soo Park[1,5], Soojin Hwang[1,5], Yoon Ki Hong[1], Gahee Choi[1], Yoon Seak Suh[1], Seung Yeop Han[1], Darae Kim[1], Jungae Jeun[2], Chun-Taek Oh[3], Sung-Joon Lee[2], Sung-Jun Han[3], Donghak Kim[1], Eun Soo Kim[1], Gilsang Jeong[4], and Kyoung Sang Cho[1,*]

Hempseed, a rich source of polyunsaturated fatty acids (PUFAs) and phytosterols, has been recognized as a potential therapeutic food used for cardioprotection, preventing platelet aggregation, and improving atopic dermatitis. Although several studies have revealed the physiological benefits of hempseed on a variety of animals, the effects of dietary hempseed intake on animal development are currently unknown. In this study, we evaluated the developmental effects of the addition of hempseed meal (HSM) to the diet of *Drosophila*. Interestingly, dietary HSM intake was shown to increase the body size of flies by increasing cell numbers, and also truncated the larval period without affecting survival rate or longevity. The oviposition of female flies was also increased by dietary HSM supplementation. Interestingly, the levels of sterols, which are precursors of ecdysone, a molting hormone, were found to be elevated in the larvae fed on HSM. Additionally, the hexane extracts of hempseed mimicked the effects of HSM on growth, developmental timing, and reproduction. Moreover, among the major nonpolar components of HSM, feeding on cholesterol but not PUFA mix or campesterol accelerated pupariation and increased body size. These results indicate that the dietary intake of HSM accelerates both body growth and developmental rates in *Drosophila* via the stimulation of cell growth and ecdysone synthesis. Additionally, nonpolar components of hempseed, such as cholesterol, might be responsible for the effects of HSM on development and reproduction.

INTRODUCTION

Hempseed is an excellent nutritional resource, which contains more than 30% oil, approximately 25% proteins, and over 20% fiber (Callaway, 2004; Hullar et al., 1999; Yazicioglu and Karaali, 1983). Hempseed oil is composed of over 80% of polyunsaturated fatty acids (PUFAs), which include essential fatty acids, linoleic acid (18:2n6), and alpha-linolenic acid (18:3n3) as major components, as well as gamma-linolenic acid (18:3n6) (Callaway, 2004). Many studies have demonstrated that these fatty acids are critically important for human health (Connor et al., 1993; Fedor and Kelley, 2009; Holub and Holub, 2004; Panza et al., 2009; Wendel and Heller, 2009). Virgin hempseed oil also contains 3922-6719 mg/kg of phytosterols (e.g., sitosterol and campesterol) (Matthäus and Brühl, 2008), which have been implicated in the prevention of cardiovascular diseases due to their ability to lower total cholesterol and low density lipoprotein-cholesterol levels in serum (Malini and Vanithakumari, 1990; Miettinen and Gylling, 2004; Patch et al., 2006). Additionally, hempseed is also a good source of high-quality protein. The amino acid profiles of hempseed proteins are comparable to those of soybean or egg whites, and harbor all components essential for human health (Callaway, 2004).

As a nutritional source, hempseed has been utilized in the human diet for thousands of years, and has recently been categorized as a valuable product in the food industries of Canada and European countries (Callaway, 2004). Hempseed can also be utilized in animal feed. Several studies have proposed hempseed as a valuable food source for farm animals, including fish (Webster et al., 2000), pigeons (Hullar et al., 1999), hens (Silversides and Lefrançois, 2005), and cattle (Hessle et al., 2008; Mustafa et al., 1999).

In addition to its utility as a food source, hempseed has been utilized for medicinal purposes for at least 3000 years in Asia (Callaway, 2004; de Padua et al., 1999; Kim and Mahlberg, 2003). A series of recent studies have demonstrated that dietary hempseed intake provides significant cardioprotective effects during postischemic reperfusion injury in rats and rabbits (Al-Khalifa et al., 2007; Prociuk et al., 2006), and the cholesterol-induced stimulation of platelet aggregation was prevented

[1]Department of Biological Sciences, Konkuk University, Seoul 143-701, Korea, [2]College of Life Science and Biotechnology, Korea University, Seoul 136-701, Korea, [3]Drug Biology Group, Institut Pasteur Korea, Seongnam 463-400, Korea, [4]Laboratory of Environmental Entomology, Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-853, Korea, [5]These authors contributed equally to this work.
*Correspondence: kscho@konkuk.ac.kr

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by a hempseed-enriched diet (Prociuk et al., 2008). Additionally, dietary hempseed oil improved clinical symptoms in patients with atopic dermatitis (Callaway et al., 2005). These benefits of hempseed may be attributable to its optimal balanced PUFAs, which include linoleic (Hashem et al., 2008), alpha-linolenic, and gamma-linolenic acids. Indeed, hempseed intake can induce significant changes in serum lipid profiles in both humans and animals (Callaway et al., 2005; Kang and Park, 2007; Karimi and Hayatghaibi, 2006; Schwab et al., 2006).

Hempseed may also be nutritious for insects. Although the effects of dietary hempseed intake in insects remain to be clearly elucidated, many insects feed on immature hempseed in the field, and some insects, including *Drosophila busckii*, have been implicated in the consumption of seeds in storage (McPartland et al., 2000). In fact, phytosterols and PUFAs, the highly enriched components of hempseed, are known to be important nutrients for insects. Phytosterols are precursors of ecdysone, which functions in the control of both molting and reproduction (Rees, 1985; 1995; Svoboda and Thompson, 1985), and PUFAs have been implicated in ethanol and cold tolerances (McKechnie and Geer, 1993; Ohtsu et al., 1993; Overgaard et al., 2005), neuroprotection (Min and Benzer, 1999), and the excitation of photoreceptors (Chyb et al., 1999) in insects.

Although several previous studies have attempted to characterize the effects of dietary hempseed intake, its nutrimental benefits have yet to be completely understood; this is particularly true in regard to the effects on animal development of a hempseed diet. In this study, we evaluated the effects of hempseed meal (HSM) on the development and reproduction of *Drosophila*. Interestingly, dietary HSM intake accelerates body growth by increasing cell numbers, increasing egg laying, and shortening the larval stage of *Drosophila*.

MATERIALS AND METHODS

Fly strains and hempseed meal (HSM)

Fly strains

The wild-type strain utilized in this study was obtained from the Bloomington *Drosophila* Stock Center (USA). In order to compare the effects of HSM on *Drosophila* development and reproduction with cornmeal-soybean standard (CTL) media, Canton S, the wild-type strain of *Drosophila melano-gaster,* was cultured on each media at 25°C.

HSM

The contents of HSM are described in Table 1. In HSM, soybean and cornmeal flour of standard control media were replaced with hempseed dregs. The hempseed dregs are the remains left after pressing the oil out of the hempseeds. To rule out the caloric effect, the calories of each medium were calculated from previous data (Hullar et al., 1999; Moe et al., 1973; Silversides and Lefrançois, 2005) and adjusted to equal levels.

Drosophila food with PUFA mix, campesterol, or cholesterol

To evaluate the effects of nonpolar hempseed components, cornmeal-soybean standard media with PUFA mix, campesterol, or cholesterol were employed. 8.66 mg/ml of PUFA Mix RM-2 (Sigma-Aldrich, USA), 7.32 μg/ml of campesterol (Sigma-Aldrich, USA) in chloroform, and 0.351 μg/ml cholesterol (Sigma-Aldrich, USA) in ethanol were added to the standard media; these are the same amounts that are contained in HSM (Callaway, 2004; Matthäus and Brühl, 2008). For the control experiments, 1.46 μl/ml of chloroform or 0.351 μl/ml ethanol was added to the media.

Table 1. Composition of the two diets fed to *Drosophila* in this study.

	Control media	HSM media
Water	6,500 ml	6,500 ml
Yellow cornmeal	475 g	-
Soybean flour	65 g	-
Hempseed dregs	-	476 g
Agar	112.5 g	112.5 g
Yeast	37.5 g	37.5 g
Light malt extract	333 g	333 g
Light corn syrup	585 ml	585 ml
Propionic acid	31.3 ml	31.3 ml
Methyl *p*-hydroxybenzonate	11.65 ml	11.65 ml
95% Ethanol	41.65 ml	41.65 ml

Measuring pupariation time

The analysis of pupariation time was conducted as follows. The wild-type embryos (0-3 h) were collected in plates containing grape juice agar (Sullivan et al., 2000), the embryo-collecting media, after which these embryos were transferred to vials containing the indicated media. In order to avoid crowding, 50 embryos were placed into each vial. After transfer, the numbers of pupa were counted every 24 h.

Measuring food intake

Food intake was measured as described previously (Edgecomb et al., 1994) with some modifications. In brief, less than 96 h after egg laying, 50 larvae were starved for 30 min and transferred into each new media with 0.05% bromophenol blue, then maintained for 10 min. The larvae were homogenized to measure the level of blue color with a spectrophotometer at 595 nm.

Measuring egg laying

Five male and female flies per group were used to measure egg laying. The 3-day-old flies reared in indicated media were placed together to allow them to mate in vials for 24 h. The mated flies were then moved onto grape juice agar plates (Sullivan et al., 2000). The number of laid eggs was counted 24 h after transfer.

Measuring survival and longevity

Measuring survival

100 wild-type embryos brought up in appropriate media were collected on grape juice agar plates. After, these embryo were then transferred to HSM or CTL media and reared at 25°C in upright standard plastic shell vials. Embryo and hatched larvae were maintained under non-crowded conditions, at 20 individuals per vial. The numbers of pupae and enclosed adult flies were counted.

Measuring longevity

To measure longevity, 20 flies were reared per vial with the appropriate media. The flies were transferred to vials containing fresh media every 3 days, and the numbers of live flies were counted. For one experimental set, a total of 100 flies were tested for each media. The longevity analysis for each sample was repeated five times.

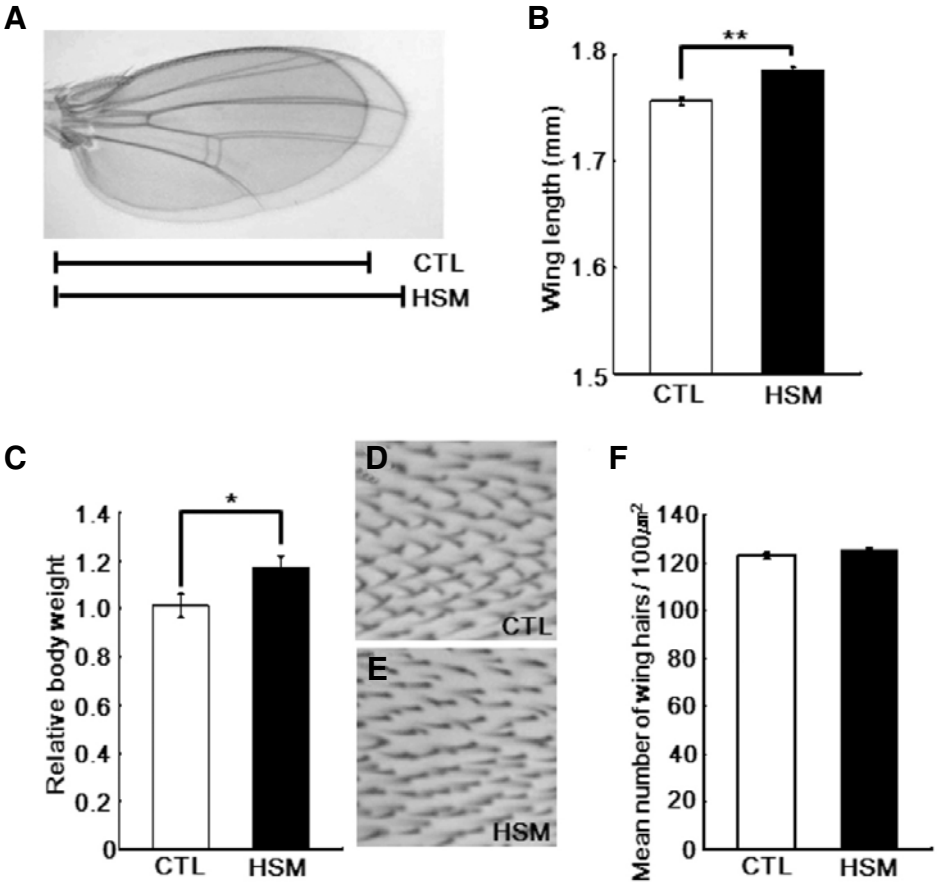

Fig. 1. The effects of an HSM diet on the growth of *Drosophila*. (A, B) Wing size comparison between the HSM-fed and CTL-fed flies. Merged photograph (A) of wings from the HSM-fed and CTL-fed flies and statistical analysis (B) of wing size showed significant increases in wing length in the HSM-fed flies as compared to the CTL-fed flies (**P < 0.001, n = 100, Student's t-test). Error bars represent ± SE. (C) Increased body weights in the HSM-fed flies compared with the CTL-fed flies (*P < 0.01, $n \geq 8$, Student's t-test). Error bars represent ± SE. (D-F) Comparing the wing hair cell density between HSM-fed and CTL-fed flies. Photographs of the wings of CTL-fed (D) and HSM-fed (E) flies and statistical analysis (F) of the wing hair cell density show no prominent differences between the wing hair cell densities of HSM-fed flies and those of the CTL-fed flies. Error bars represent ± SE. CTL, cornmeal-soybean standard media; HSM, hempseed meal media.

Measuring body size

Measuring wing length

Wings from adult flies were dissected in PBS and mounted on Gary's Magic Solution. Then, wing length was measured with an Axio Vision AC 4.5.0.0. microscope program (Carl Zeiss, Germany).

Measuring body weight

To measure body weight, the 50 embryos were maintained in the indicated media. These embryos were reared in appropriate media until they matured into adult flies. Then, 50 male flies were gathered in 1.5 ml microcentrifuge tubes, and their weights were measured with an XT 220A Analytical Balance (Precisa, Switzerland).

Sterol assay

The sterol levels in larvae were quantified via a previously published method (Fluegel et al., 2006) using an Amplex Red cholesterol assay kit (Molecular Probes, USA). In brief, 30 wandering third-instar larvae were collected and washed prior to weighing and homogenization in 150 mM NaCl, 2 mM EGTA, and 50 mM Tris at a pH of 7.5. The homogenates were spun at 5,000 rpm for 5 min to pellet cuticle debris, and the supernatants were utilized for Amplex Red reactions for 30 min at 37°C. Fluorescence was measured with a fluorescence spectrophotometer equipped with a 560/585 nm filter set.

Triglyceride assay

Total body triglycerides and protein were measured as previously described, with some modifications (Teleman et al., 2005). 15 third-instar larvae were homogenized in 600 μl of lysis solution (150 mM NaCl, 10 mM Tris-HCl, 0.1% Triton X-100, pH 8.0). The homogenate solution was then filtered with a 0.45 μm filter (Satorius, UK). The triglyceride concentrations of the solutions were measured using a Triglyceride Assay Kit (Thermo, USA) in accordance with the manufacturer's recommendations. Approximately 7 μl of filtered samples were mixed with 150 μl of triglyceride assay solution in 96-well plates. After 10 min of incubation at 37°C, the absorbance was measured at 500 nm with a fluorescence plate reader (Molecular Device, USA). The protein concentrations of the filtered samples were measured with a Protein Assay Kit (iNtRON, Korea). The plotted values were triglyceride levels normalized to protein levels.

Extraction of hempseed

Washed hempseeds were dried and ground using an electric grinder. Nonpolar and polar components were extracted from the ground hempseeds using hexane and distilled water, respectively. First, 300 ml hexane was added to 100 g of ground hempseed, stirred for 24 h, and filtered through 0.45 μm Whatman nylon membrane filters to separate the residues and extracted solutions. 30 ml of nonpolar components were extracted in liquid form. The filtered residue was freeze-dried and mixed with 300 ml of distilled water for extraction via a similar method. This distilled water extract was dried and dissolved with 50 ml of distilled water. All filtered extracts were evaporated at 35°C using a rotary evaporator (EYELA N-1000, Japan). In order to remove any remaining solvents, the extracts were re-evaporated for 10 min at 70°C. These extracts were utilized in further

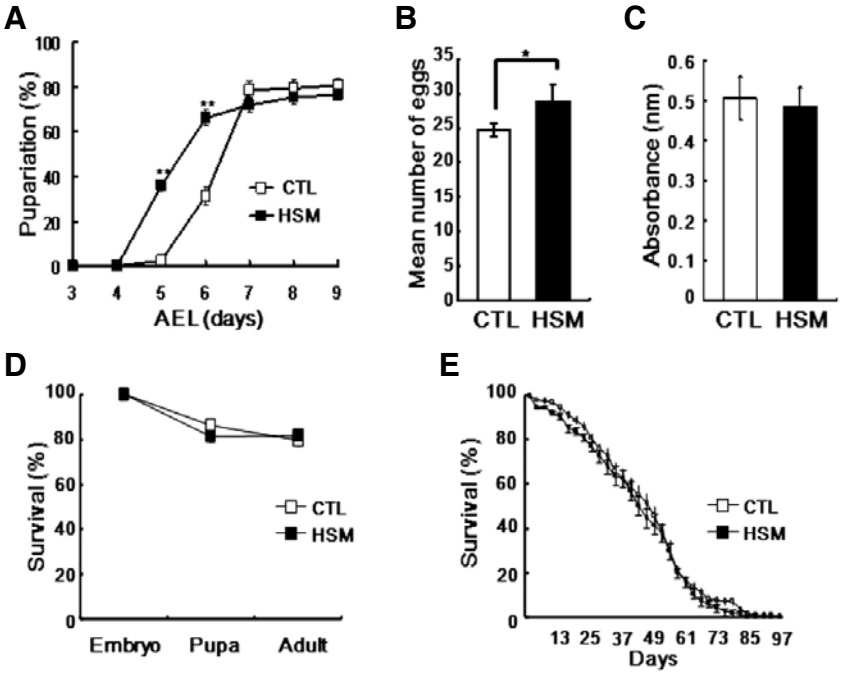

Fig. 2. The effects of dietary HSM intake on *Drosophila* development and reproduction. (A) Growing up in HSM, the flies had shorter pupariation times after egg laying as compared to the CTL-fed flies (***P* < 0.001, *n* = 300, Student's *t*-test). Error bars represent ± SE. (B) The egg laying ability of HSM-fed flies was increased by approximately 16% over that of CTL-fed flies (**P* < 0.05, *n* = 35, Student's *t*-test). Error bars represent ± SE. (C) There were no marked differences in the food intake of HSM- and CTL-fed flies (*n* = 270). Error bars represent ± SE. (D, E) Survival rates of each developmental stage (*n* = 13) (D) and longevity (*n* = 5) (E) were unaltered in the HSM-fed flies as compared with the CTL-fed flies. Error bars represent ± SE. AEL, after egg laying; CTL, cornmeal-soybean standard media; HSM, hempseed meal media.

experiments.

RESULTS

Intake of hempseed meal (HSM) increases body size

In order to verify the possible effects of dietary HSM intake on *Drosophila* development, flies were reared in HSM throughout their lifetimes, and their growth rates were compared to the cornmeal-soybean standard media (CTL)-fed flies (Fig. 1). First, the wing sizes of both the HSM- and CTL-fed flies were compared; interestingly, the wings of HSM-fed flies were larger than those of CTL-fed flies (Figs. 1A and 1B). Second, body weights were also shown to have increased when the flies were reared in HSM media (Fig. 1C). Next, we attempted to determine whether increased body size was the result of increased cell numbers or enlarged cell sizes by counting the numbers of wing hair cells in the limited wing region. As shown in Figs. 1D-1F, no prominent changes were noted in the hair cell density, which suggests that the cell size in the wings of the HSM-fed flies was unaltered. Therefore, the observed increases in body size might have been due to increased cell numbers.

Intake of HSM accelerates pupariation and egg laying

It has been reported in previous studies that the final body size depended on both the larval growth rate and the duration of each larval stage (Caldwell et al., 2005; Mirth et al., 2005; Son et al., 2008), and that increased body size was related with lengthened duration of the larval stage (Caldwell et al., 2005; Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). Therefore, we attempted to determine whether increased body size was the result of a prolonged larval stage. However, the larval stage of HSM-fed flies was shortened by 24 h as compared to the CTL-fed flies (Fig. 2A). As the total calories of each food were calculated and adjusted to be identical, this phenomenon was not the result of different calories, but rather of differing nutritional components. This means that hempseed may contain some component(s) that may prove to be responsible for accelerations of larval growth and reductions of developmental time.

Next, the effects of HSM feeding on *Drosophila* reproduction were evaluated by comparing the numbers of eggs laid by the HSM- and CTL-fed females. As shown in Fig. 2B, the female flies fed with HSM laid approximately 16% more eggs than the flies fed with CTL media, thus suggesting that HSM also harbors some nutritional component(s) that promote the reproduction of *Drosophila*. In order to rule out the possibility that flies reared in HSM media take in more food than flies in CTL media, the intakes of each were quantified. As shown in Fig. 2C, food intakes in each group did nto differ profoundly, thereby indicating that the effects of HSM on *Drosophila* development and reproduction were not the results of increased food intake. However, unlike pupariation time, the survival rates and longevity of HSM-fed flies did not differ from those of the CTL-fed flies (Figs. 2D and 2E).

Intake of HSM increases sterol levels

As ecdysone has been shown to be involved in *Drosophila* oogenesis as well as development (Fletcher and Thummel, 1995), the effects of HSM on pupariation time and fecundity are, presumably, a consequence of increased ecdysone levels. Additionally, sterol is an initial precursor of ecdysone in *Drosophila* and all other insects (Huang et al., 2008). Because insects cannot synthesize sterol from acetate *de novo*, they must first acquire either cholesterol or several other side-chain alkylated plant sterols from food (Clark and Block, 1959). Therefore, we attempted to determine whether the observed elevated growth rates and fecundity in the HSM-fed larvae and flies were the consequence of increased levels of sterols. According to the sterol assay with total larva extract, we determined that the sterol levels were increased by approximately 30% in the HSM-fed larvae (Fig. 3A). This indicated that increased sterol levels might be responsible for the elevated levels of ecdysone, and thus might be implicated in the acceleration of the developmental rate and the egg laying rate.

Next, we compared the lipid levels of HSM-fed larvae with those of the CTL-fed larvae, in order to assess whether or not

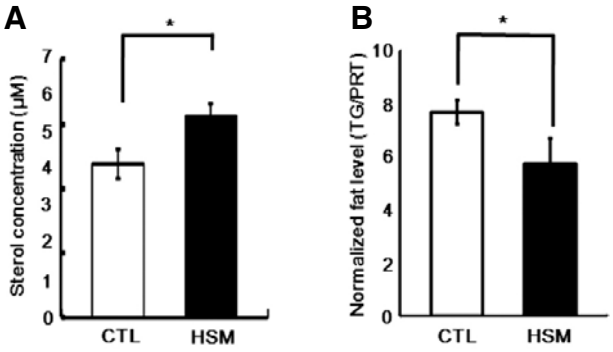

Fig. 3. The effects of a HSM diet on the level of sterol and fat. HSM-feeding increased concentration of sterol (*$P < 0.05$, $n = 9$, Student's *t*-test) (A) and reduced the levels of triglycerides (TG) (*$P < 0.05$, $n = 7$, Student's *t*-test) (B). Error bars represent ± SE. CTL, cornmeal-soybean standard media; HSM, hempseed meal media; PRT, protein; TG, triglyceride.

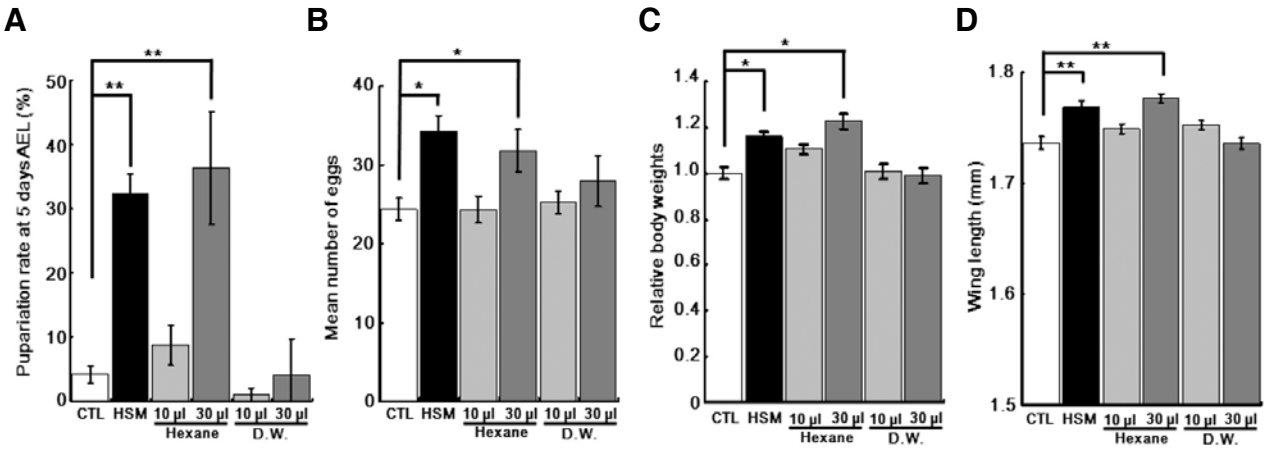

Fig. 4. The effect of crude extracts of hempseed by hexane and distilled water on *Drosophila* development and reproduction. To test these effects, flies were reared in standard media with the indicated amounts of each hempseed extract. (A) The hexane extracts evidenced effects similar to those of HSM on larval development, but distilled water extract did not (**$P < 0.001$, $n \geq 200$, Student's *t*-test). Error bars represent ± SE. (B) The hexane extract evidenced effects similar to those of HSM on egg laying, but distilled water extract did not (*$p < 0.05$, $n \geq 20$, Student's *t*-test). Error bars represent ± SE. (C, D) Increased body weight (C) and wing length (D) in hexane extract-fed flies compared with CTL- or distilled water extract-fed flies (*$P < 0.05$, $n \geq 3$, Student's *t*-test) (**$P < 0.001$, $n \geq 50$, Student's *t*-test). Error bars represent ± SE. AEL, after egg laying; CTL, cornmeal-soybean standard media; HSM, hempseed meal media; D.W., distilled water.

elevated lipid levels could influence larval development by increasing calories. As shown in Fig. 3B, the lipid levels were not increased by feeding with HSM, but rather were reduced; this indicated that elevated lipid levels were not a relevant issue in the effects of HSM on *Drosophila* development.

Hexane extract of hempseed recapitulate HSM effect

To understand the characteristics of hempseed's components that may affect the development and reproduction of *Drosophila*, the crude hempseed extracts were prepared with hexane and distilled water, and the effects of standard media with each extract on development and reproduction were evaluated. The hexane extract exerted effects similar to those of HSM on both development (Figs. 4A, 4C, and 4D) and oviposition (Fig. 4B). By way of contrast, the aqueous extract exerted no accelerating effects on the growth or reproduction of *Drosophila* (Fig. 4). These results indicate that the nonpolar components, such as sterols or fatty acids, might be responsible for the effects of HSM on *Drosophila* development and reproduction.

Cholesterol, but not PUFA or campesterol, accelerates pupariation and increases body size

Next, we evaluated the effects of three representative nonpolar components of hempseed-PUFA, phytosterol, and cholesterol-on the pupariation and body growth of *Drosophila*. To achieve this, PUFA mixtures similar to hempseed components and campesterol, the second most abundant phytosterols in hempseed (Matthäus and Brühl, 2008), were employed to assess the effects of PUFA and phytosterol.

As is shown in Fig. 5A, feeding with 0.351 μg/ml cholesterol, the same amount as contained in HSM, accelerated larval pupariation. However, counter to our expectations, the larvae fed on PUFA mix or campesterol grew rather slowly relative to the controls (Figs. 5B and 5C). Additionally, cholesterol feeding also increased body weight (Fig. 5D) and wing length (Fig. 5E), whereas PUFA mix and campesterol diet supplementation treatments exerted no effects on body growth (Figs. 5D and 5E). These results indicate that the cholesterol in HSM is responsible, at least in part, for the effects of HSM on *Drosophila* development.

DISCUSSION

Although hempseed has been recognized for thousands of years as an excellent source of nutrition, the effects of dietary hempseed intake have yet to be fully understood, owing largely to the politics prohibiting its cultivation (Callaway, 2004). In this study, we evaluated the effects of hempseed meal (HSM),

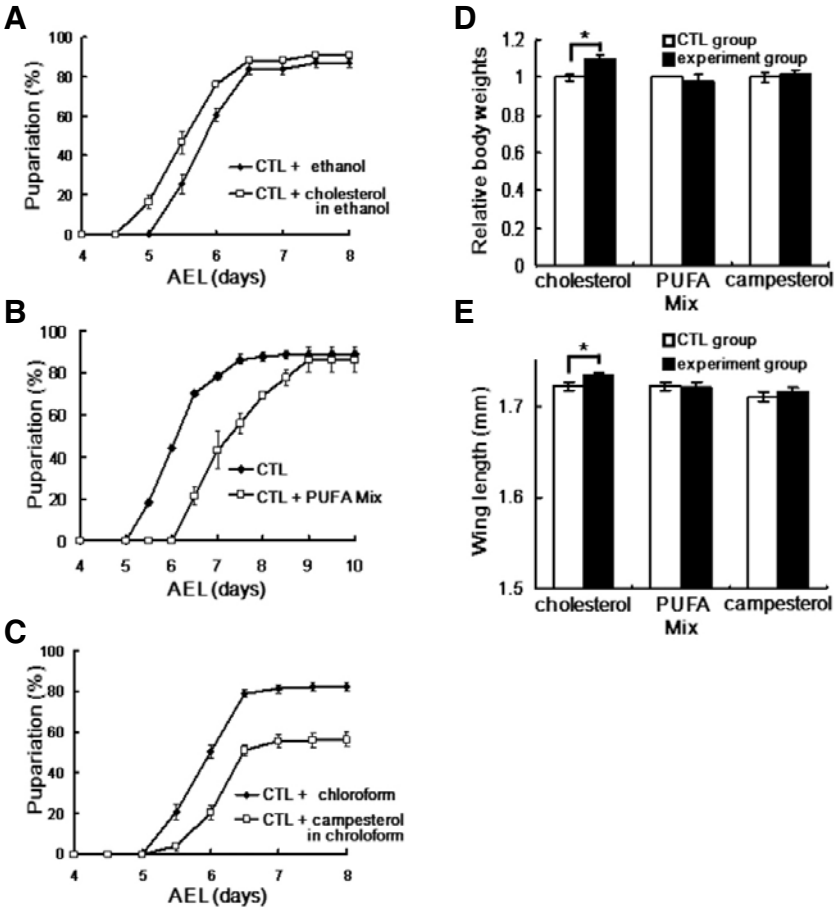

Fig. 5. The effect of cholesterol, PUFA mix, and campesterol on *Drosophila* development. To test the effects, files were reared in standard media with 0.351 μg/ml of cholesterol, 8.66 mg/ml of PUFA mix, or 7.32 μg/ml of campesterol-the same amounts as in HSM. (A-C) Comparing the pupariation time between larva reared in indicated media with appropriate control media. (A) The cholesterol showed similar but less effect with HSM on larval development (n = 500). On the contrary, PUFA mix (B) and campesterol (C) showed delayed pupariation time (n = 100) (n = 500). Error bars represent ± SE. (D, E) Increased body weight (D) and wing length (E) in cholesterol-, but not PUFA mix- or campesterol-fed flies (*P < 0.01, $n \geq 3$, Student's t-test) (*P < 0.01, $n \geq 35$, Student's t-test). Error bars represent ± SE. CTL, cornmeal-soybean standard media; CTL + ethanol, CTL with 0.351 μl/ml of ethanol; CTL + cholesterol in ethanol, CTL with cholesterol dissolved in ethanol; CTL + PUFA mix, CTL with PUFA Mix; CTL + chloroform, CTL with 1.46 μl/ml of chloroform; CTL + campesterol in chloroform, CTL with campesterol in chloroform; CTL group, flies reared in appropriate control media; experimental group, flies reared in indicated media; cholesterol, cholesterol-fed flies; PUFA Mix, PUFA mix-fed flies; campesterol, campesterol-fed flies.

which contains hempseed dregs, on *Drosophila* development and reproduction. Dietary HSM intake shortened the time in larval stage and increased body size, growth, and egg laying without affecting survival rates during development and adult longevity as compared to soybean and cornmeal intake (Fig. 1). The effects on development and oviposition were recapitulated by the nonpolar extract of hempseed (Fig. 4). These results demonstrate that hempseed dregs provide sufficient nutrition for *Drosophila*, and still contain enough bioactive molecules to influence the physiology of *Drosophila* despite the extraction of the majority of hempseed oil.

The data obtained in this study demonstrated that cholesterol is responsible, at least in part, for the effects of HSM on *Drosophila* development. As a precursor of ecdysone, cholesterol is a critically important nutrient factor in insects (Huang et al., 2008). Unlike vertebrates, insects are unable to synthesize sterols *de novo* from small molecules, such as acetate, and must therefore obtain sterols from their diet (Rees, 1995). Therefore, dietary cholesterol uptake would be expected to influence insect development. Indeed, hempseed oil contains reasonable quantities of cholesterol (34 mg/kg) (Matthäus and Brühl, 2008), whereas corn and soybean oil do not (RDA, 2006). Thus, cholesterol in HSM may be converted into ecdysone in HSM-fed larva, and this increased ecdysone content may influence the pupariation time of the larva. Moreover, cholesterol has previously been implicated in ovarian development in the house fly (*Musca domestica* L.) (Monroe, 1959; Robins and Shortino, 1962), thus suggesting that cholesterol is also responsible for the effects of HSM on the reproduction of *Drosophila*. In the meantime, we determined herein that cholesterol uptake increases body size. It would be interesting to determine whether the effect of HSM is diminished in mutants of *DHR96* or the Niemann-Pick C1-like 1 gene, which mediates the intestinal uptake of dietary cholesterol (Ge et al., 2008; Horner et al., 2009; Wang, 2007).

Although cholesterol affects larval growth, our data demonstrated that all effects of HSM on the larval development do not rely on cholesterol. Indeed, the effect of cholesterol feeding on pupariation time and body growth is far less profound than that of HSM feeding, even though the same amount of cholesterol contained in HSM was used (compare Figs. 1A-1C and Fig. 2A with Figs. 5D, 5E, and Fig. 5A, respectively). These results indicate that HSM contains components other than cholesterol that affect insect development. Some possible candidates include phytosterols and PUFA, high contents of which are involved in HSM (Matthäus and Brühl, 2008). However, our studies demonstrated that feeding with PUFA mix or campesterol, the second most abundant phytosterol in hempseed oil, did not accelerate pupariation time, but rather delayed it. Furthermore, these treatments did not affect *Drosophila* growth. As PUFA mix contains the majority of PUFAs that are involved in HSM, we can exclude the possibility that certain PUFAs are responsible for the effects of HSM on the growth of *Drosophila*. On the other hand, hempseeds contain at least 11 phytosterols, with the exception of campesterol (Matthäus and Brühl, 2008). As a precursor of ecdysone, phytosterols that can be converted into cholesterol are essential for growth, development, and reproduction (Rees, 1985; 1995; Svoboda and Thompson, 1985). As

hempseed has a high phytosterol content (Matthäus and Brühl, 2008), hempseed intake may increase sterol levels in the body, which might in turn elevate the levels of ecdysone. Consistent with this notion, previous studies have demonstrated that sitosterol, one of the most abundant phytosterols in hempseed, supports growth and development better than cholesterol (Cooke and Sang, 1970; Svoboda et al., 1989). Moreover, in our study, the sterol levels of HSM-fed larvae were higher than those of the CTL-fed larvae, and the nonpolar components, which contain sterols, recapitulated the effects of HSM on *Drosophila* development and reproduction. Therefore, despite the fact that campesterol evidenced no positive effects on the shortened larval growth and increased body size, we are unable to dismiss the possibility that other phytosterols, such as sitosterol, are responsible for the effects of HSM on *Drosophila* development.

According to the results of a previous study, in which it was demonstrated that ecdysone suppressed body size growth (Colombani et al., 2005) and that sterols are precursors of ecdysone (Rees, 1995), the elevated levels of sterols in HSM-fed larvae are presumed to result in reductions in body size. However, on the contrary, HSM was shown to increase body size (Fig. 1) and also elevated sterol levels (Fig. 3), which indicates that one or more other factors may promote larval growth to compensate for the growth inhibition effect of ecdysone. In this study, we determined that cholesterol both shortens larval periods and increases body size. As increased body size is the consequence of increased cell numbers, cholesterol may affect cell division during insect development. Then, how does cholesterol accelerate cell division? In animals, cholesterol supports lipid digestion and absorption via the formation of chylomicrons, and is also involved in the delivery of triglycerides to different organs (Fox, 2009). Therefore, it could influence the growth of *Drosophila* by enhancing the lipid metabolism pathway in the cells. Alternatively, cholesterol has been implicated in insulin receptor signaling for metabolic control (Parpal et al., 2001). The insulin signaling pathway can increase body size in a cell autonomous manner, and operates as an ecdysone antagonist in the determination of final body size in *Drosophila* (Caldwell et al., 2005; Colombani et al., 2005). Therefore, the increased cholesterol levels resulting from HSM feeding may also activate this pathway to inhibit the body size reductions resulting from increases in ecdysone levels. Collectively, our data indicates that HSM feeding accelerates both body growth and developmental rates via the stimulation of cell-growth signaling pathways, and ecdysone synthesis by increasing sterol levels in the body. As cell growth signaling and the level of steroid hormones are also critical determining factors in body size and the timing of developmental processes in mammals, it would be interesting to attempt to determine whether HSM can also influence mam-malian development.

The data obtained in this study implicate HSM as a prominent source of nutrients, which can stimulate both body growth and developmental rates in *Drosophila*. These observations emphasized the importance of the nutritional environment in regard to proper development, as determined by the interaction between growth rates and developmental timing.

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