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Grafted Larval Age as a Factor Affecting Honeybee (*Apis mellifera*) Queen Cell Acceptance and Morphometric Characteristics

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Key words
Larvae grafting, Larval age, Morphometric characteristics, Larval acceptance, *Apis mellifera*

ABSTRACT

Queen is the prime entity of the honeybee colony, which determines the fate of the colony's productivity. The methods used for rearing honeybees are crucial to their success. This study was conducted in the spring of 2020 and 2021 to examine how queen cell acceptance rates and morphometric features i.e., length and width of head, thorax, wings, and body were affected by grafted larval age. For the rearing of queen bees, one-day-old, two-day-old, and three-day-old larvae were used in 11 starter hives from one breeding hive. The results exhibited a significant influence of grafted larval age on larval acceptance and morphometric features in both years. The highest acceptance rate was observed for one-day-old grafted larvae (81.11% in each study year), whereas the lowest was for three-day-old larvae (6.67% in 2020 and 7.78% in 2021). The highest mean larval acceptance for both of the years was also recorded in one-day-old grafted larvae i.e., 24.31% in the year 2020 and 24.30% in the year 2021. Whereas the lowest was recorded in three-day-old larvae i.e., 2.00% in 2020 and 2.30% in 2021. The highest body weight, head length, head width, thorax length, thorax width, wing length, wing width, and queen body length for both of the years was recorded in one-day-old grafted larvae i.e., 159.20 mg, 3.27 mm, 3.36 mm, 3.91 mm, 4.14 mm, 10.59 mm, 3.45 mm, and 21.33 mm in the year 2020 respectively and 156.90 mg, 3.26 mm, 3.37 mm, 3.96 mm, 4.18 mm, 10.63 mm, 3.50 mm, and 21.03 mm in the year 2021 respectively. Whereas the lowest was recorded in three-day-old larvae in both of the years. It is concluded that one-day-old grafted larvae are suitable for the production of healthier queen bees. These findings can provide direction for attempts to improve the quality of honeybee queens in profitable queen raising, which is necessary for resolving difficulties that are at the root of the increasing number of queen failures in the apiculture industry.

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INTRODUCTION

Pollination is a crucial factor in the agriculture industry and acts as a key framework for crop yield. Bees especially honeybees are the most important ecosystem services provider like pollinators of various fruits (Benachour and Louadi, 2013; Gurmani *et al*., 2016; Akram *et al*., 2022), vegetables (Amoako and Yeboah-

Gyan, 1990; Negi *et al*., 2020), and agronomic crops (Rizzardo *et al*., 2012; Abbasi *et al*., 2021; Khan and Ghramh, 2021) throughout the world. In each colony of honeybees, a queen is considered the most critical individual (Moore *et al*., 2015), on which the success of the colony depends (Winston, 1987). Moreover, only the queens have the ability to lay eggs either sterile or fertile, resulting in overlapping generations of brood throughout the year and releasing pheromones that prevent the worker bees from developing new queens (Winston *et al*., 1981; Delaney *et al*., 2011). The primary task of the queen is to produce daughter workers for foraging and caring for the brood, eggs, larvae, and pupae, as well as drones for genetic variation among colonies by mating with virgin queens (Delaney *et al*., 2011; Ozbakır, 2023). One of the most important demands by the colony placed on the queen for nonstop breeding, survival and reproductive output, highlights the relevance of the queen's wellbeing to the colony's success. As a healthy queen increases the amount of revenue collected from the selling of additional bees, some hive products (including propolis, honey, and royal jelly), and pollination services, beekeepers prefer to have healthy queens. The failure of the queen can cause the mortality of the honeybee colony (vanEnglesdorp *et al*., 2010). Therefore, high-quality queen bees are essential for colony productivity and high quantity and quality production of bee hive products (Yu *et al*., 2022).

In beekeeping, the artificial rearing of the queen honeybee is crucial to regularly strengthen the colonies, improve their genetic features, upsurge the number of workers, enhance the production of honey, and reduce swarming tendencies (Adgaba *et al*., 2019). Nowadays, the population of honeybees is declining due to various biotic and abiotic factors (Stankus, 2008; Panziera *et al*., 2022). The pathogen, parasites, and *Varroa destructors* are considered the most important factors linked with poor colony health and responsible for colony losses. Additionally, diseased or lost queens are regarded as one of the most important and main causes of colony losses. Among other factors, the poor queen is the primary and important problem in beekeeping, sometimes leading to colony failure in peak seasons. Notably, commercial progressive beekeepers found that the failure of the queen honeybee is the most crucial factor in colony collapse (Kulhanek *et al*., 2017). To determine the health of their queens, beekeepers examine the colony and note numerous indicators associated with the queen's reproductive output. There are several factors that affect the quality production of the queen i.e., grafting of larvae, grafting techniques, number of the grafted larvae, age of the larvae, health of the larvae, hands-on practice of grafter, environmental conditions, and grafting materials (Njeru *et al*., 2017; Okuyan and Akyol, 2018; Güneşdoğdu and Şekeroğlu, 2020; Yi *et al*., 2021). So, most beekeepers replace the queens each year because of their importance and colony survival. However, beekeepers observed the failure of the young and newly mated queen into the colonies, but unfortunately, the causes are still unknown.

Some of the morphological characters i.e., length and width of head, thorax and wings and body weight are crucial in adult insects (Fischer and Maul, 1991; Gilley *et al*., 2003; Kahya *et al*., 2008) and among these, few have been found positively correlated with the fecundity or reproductive success of honeybee queen (Woyke, 1971; Nelson and Gary, 1983). Moreover, Kaftanolu *et al*. (1988) reported that the genotype, nutritional parameters, raising practices, time of rearing, grafted larval age, and grafted larval quantity in each cell builder colony (starter colony); all have an influence on the quality production of queen bees. Beekeepers who want to produce queen bees can easily change the amount and age of grafted larvae. They can produce the maximum superior quality queen bees by using the right quantity of grafted larvae. The quality of the queen bee will decrease if there are more grafted larvae than the optimum (Korkmaz, 2005). It has been reported that the age of the grafted larvae has the greatest impact on the quality of queen bees (Woyke, 1967; Vaziritabar and Esmaeilzade, 2018). It has been found that queen honeybees can be produced from up to three-day-old larvae (Mahbobi *et al*., 2012); however, this method is not always successful because the quality of queen bees declines as the age of grafted larvae increases. Mahbobi *et al*. (2012) found that queen bees produced from one-day-old grafted larvae are heavier than queen bees produced from two and three-days-old grafted larvae, similarly, queen bees produced from two-day-old grafted larvae are heavier than those produced from three-day-old grafted larvae. The age of the grafted larvae also has a significant influence on the acceptance rate of the queen bee cell (Okuyan and Akyol, 2018).

The influence of grafted larval age on the acceptance rate and morphometric properties of queen honeybees has been the subject of a significant amount of research that has been published on a worldwide basis (Mahbobi *et al*., 2012, 2014; Njeru *et al*., 2017; Okuyan and Akyol, 2018; Vaziritabar and Esmaeilzade, 2018). The literature is little or scarce from Pakistan. The current experiment was designed to measure how different morphometric characteristics (head length, head width, thorax length, thorax width, wing length, wing width, queen weight, and length) and larval acceptability after grafting were affected by the age of the grafted larvae.

MATERIALS AND METHODS

The experiment was carried out during the queen breeding season of the year 2020 and year 2021 at the Honeybee Research Institute (HBRI: 33°40'31"N 73°07'34"E; 508 m above sea level), National Agricultural Research Centre (NARC), Islamabad, Pakistan. In this experiment, one breeding colony of exotic honeybee *Apis mellifera ligustica* was used, headed by freshly reared queen from the grafting technique. For queen rearing through cup grafting method (Rafique *et al*., 2019), nine cell starter hives were used. As primer substrate, fresh royal jelly was used during larvae grafting that was obtained from the newly developed queen cells. To anesthetize reared queen bees, CO_2 gas at the rate of two bubbles per second for one minute was provided for easy measurement of the physical parameters of the queens (Khan *et al*., 2022). For measuring the morphometric characteristics i.e., head width, head length, thorax width, thorax length, wing width, wing length, and body length of queen, a digital vernier caliper with sensitivity of 0.01 mm was used.

Queen rearing

During the process of rearing queens, the breeder queen bee was removed from the breeding hive and placed in a queen isolator with a capacity of one frame. This allowed the queen rearing hive to receive larvae that were exactly one, two, and three days old. Frames in the queen isolator were replaced with new frames daily for three consecutive days. All frames were labeled with dates to indicate their period in the isolator. About 270 young larvae of worker bees (90 larvae per distinct age) were taken and grafted into nine grafting frames (30 larvae each). Ten larvae of each age (treatment) in each grafting frame were used randomly with nine replicated grafting frames. Ten larvae of each age were placed on a single bar in the grafting frame and properly tagged on each bar. Overall, nine cell builder five frame nucleus colonies (with two feed frames at 1st and 5th position, two sealed worker brood frames at 2nd and 4th position, grafted frame at 3rd position in the middle, and 2 to 3 frame worker nurse bees were shaken) were prepared two h prior to grafting process with closed gate for 24 to 48 h (Al-Fattah *et al*., 2011). On 3rd day, the acceptance was recorded for each age of grafted larvae. On 8th, 9th, and 10th days of grafting (respectively kept for distinct ages of grafted larvae), matured queen cells were removed from the grafting bar/frame and shifted individually in queen nursery cages along with 2-3 nurse bees having 10-gram candy (1:4, Honey: Sugar powder). On 12th day, after the emergence of queens, these queen nursery cages were shifted to queen rearing laboratory for the evaluation of morphometric characteristics.

Data recording

All queens were anesthetized with CO_2 at two bubbles/second for one minute for easy measurement of the physical parameters of the queens without damaging them (Okuyan and Akyol, 2018; Khan *et al*., 2022). Initially, their body weight (hatching weight in mg) immediately after anesthetizing was weighed through digital balance at 0.01g sensitive scale (Model: HOCHOICE - HC6002X; China) followed by other parameters including body length, head length and width, thorax length and width, wings length and width (in mm) measured with a digital vernier caliper at 0.01 mm sensitive scale (Model: Ahead-09- 154A, China). After 72 h of grafting, larvae acceptance were calculated by using the formula (Okuyan and Akyol, 2018).

$$\text{Rate of accepted larvae (RAL)} = \frac{Accepted\ larvae}{Total\ grafted\ larvae} \times 100$$

Statistical analysis

To compare the means, one-way analysis of variance (ANOVA) with post-hoc Duncan's multiple range test was used by using a computer-based statistical software SPSS version 26. Graphs were prepared on sigma plot version 16.0.

RESULTS

Larval acceptance

Table 1 shows a significant effect ($p< 0.05$) of grafted larval age on larval acceptance throughout the queen-raising seasons in 2020 and 2021. The highest acceptance rate was observed for one-day-old grafted larvae (81.11% in each study year), followed by two-day-old larvae (34.44% in 2020 and 40.00% in 2021) and three-day-old larvae (6.67% in 2020 and 7.78% in 2021) (Fig. 1).

Table I. Aptness of grafted larval age 72 h after grafting on acceptance during 2020 and 2021 queen-raising seasons.

Age of grafted larvae	Larval acceptance	
	2020 (N=30)	2021 (N=30)
One day	24.3 ± 1.2a	24.3 ± 0.88a
Two days	10.3 ± 0.8b	12.0 ± 0.57b
Three days	2.0 ± 0.57c	2.3 ± 0.88c
Results of one-way ANOVA		
F-value	9.49	6.02
DF	5	5
P-value	<0.0001	0.001

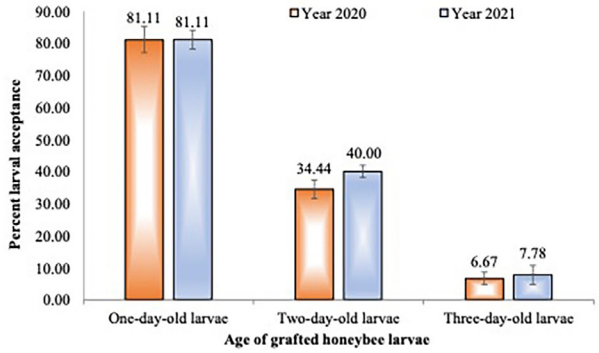

Fig. 1. Comparison of larval acceptance affected by age of grafted larvae 72 h after grafting during 2020 and 2021 queen-raising seasons.

Morphometric characteristics

There was a statistically significant difference between one day old, two days old, and three days old grafted larvae in terms of body weight of the queen, head width, head length, thorax width, thorax length, wing width, wing length, and body length of the queens during the year 2020. Similarly, in the year 2021, a statistically significant difference was also found between one day old, two days old, and three days old grafted larvae in terms of body weight of queen, head width, head length, thorax width, thorax length, wing width, wing length, and body length of the queens (Table II).

In the years 2020 and 2021, the higher queen weight was recorded for one day old grafted larvae (159.20 mg and 156.90 mg, respectively), whereas the lowest was for three-day-old grafted larvae (112.35 mg and 107.10 mg, respectively) (Table II, Fig. 2A). Similar trends were also recorded in all the studied morphometric characteristics. The maximum head width during the years 2020 and 2021 was recorded for one day old grafted larvae (3.36 mm and 3.37 mm, respectively), whereas the minimum for three-day-old larvae (1.90 mm and 1.85 mm, respectively) (Table II, Fig. 2B). In the years 2020 and 2021, the maximum head length was recorded for one-day-old grafted larvae (3.27 mm and 3.26 mm, respectively) whereas the lowest was for three-day-old grafted larvae (2.48 mm and 2.35 mm, respectively) (Table II, Fig. 2C). The maximum thorax width during the years 2020 and 2021 was recorded for one-day-old grafted larvae (4.14 mm and 4.18 mm, respectively) whereas the minimum for three-day-old larvae (2.10 mm and 1.81 mm, respectively) (Table II, Fig. 2D). In the years 2020 and 2021, the maximum thorax length was recorded for one-day-old grafted larvae (3.91 mm and 3.96 mm, respectively), whereas the lowest was for three-day-old grafted larvae (1.70 mm and 1.70 mm, respectively) (Table II, Fig. 2E).

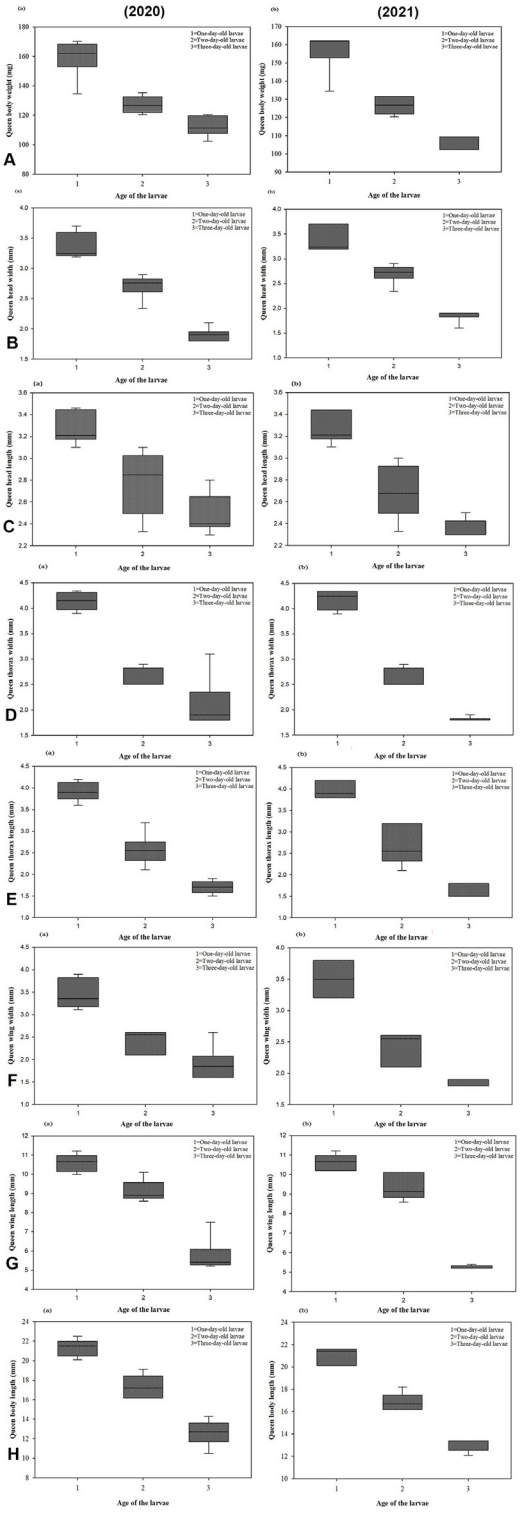

Fig. 2. Body weight (A), head width (B), head length (C), thorax width (D), thorax length (E), wing width (F), wing length (G), body length (H) of queens produced from three different ages of grafted larvae during 2020 and 2021.

Table II. Morphometric characteristics of *Apis mellifera* queens affected by age of grafted larvae.

Morphological characteristics	One day old		Two days old		Three days old		p-value
	2020	2021	2020	2021	2020	2021	
W (mg)	159.20±5.20a	156.90±4.50a	127.23±2.29b	126.60±1.89b	112.35±2.76c	107.10±1.49c	≤0.0001 (2020) ≤0.0001 (2021)
HW (mm)	3.36±0.08a	3.37±0.10a	2.71±0.07b	2.70±0.07b	1.90±0.04c	1.85±0.05c	≤0.0001 (2020) ≤0.0001 (2021)
HL (mm)	3.27±0.59a	3.26±0.05a	2.78±0.11b	2.68±0.10b	2.48±0.07b	2.35±0.03c	≤0.0001 (2020) ≤0.0001 (2021)
TW (mm)	4.14±0.07a	4.18±0.07a	2.61±0.07b	2.61±0.07b	2.10±0.20c	1.81±0.01c	≤0.0001 (2020) ≤0.0001 (2021)
TL (mm)	3.91±0.08a	3.96±0.07a	2.56±0.14b	2.66±0.18b	1.70±0.05c	1.70±0.63c	≤0.0001 (2020) ≤0.0001 (2021)
WW (mm)	3.45±0.13a	3.50±0.11a	2.41±0.10b	2.41±0.10b	1.90±0.15c	1.83±0.02c	≤0.0001 (2020) ≤0.0001 (2021)
WL (mm)	10.59±0.18a	10.63±0.16a	9.11±0.22b	9.33±0.26b	5.73±0.35c	5.25±0.03c	≤0.0001 (2020) ≤0.0001 (2021)
QBL (mm)	21.33±0.35a	21.03±0.29a	17.35±0.46b	16.86±0.33b	12.61±0.53c	13.06±0.22c	≤0.0001 (2020) ≤0.0001 (2021)

W, weight; HW, head width; HL, head length; TW, thorax width; TL, thorax length; WW, wing width; WL, wing length; QBL, queen body length.

The maximum wing width during the years 2020 and 2021 was recorded for one day old grafted larvae (3.45 mm and 3.50 mm, respectively), whereas the minimum for three days old larvae (1.90 mm and 1.83 mm, respectively) (Table II, Fig. 2F). In the years 2020 and 2021, the maximum wing length was recorded for one day old grafted larvae (10.59 mm and 10.63 mm, respectively), whereas the lowest was for three days old grafted larvae (5.73 mm and 5.25 mm, respectively) (Table II, Fig. 2G). In the years 2020 and 2021, the higher queen body length was recorded for one day old grafted larvae (21.33 mm and 21.23 mm, respectively), whereas the lowest was for three days old grafted larvae (12.61 mm and 13.06 mm, respectively) (Table II, Fig. 2H).

DISCUSSION

The ability to get a high queen cell acceptance rate is the most important factor in effective queen raising. Queen cell acceptance depends on several factors i.e., rearing methods, rearing sequence, developmental stage, strength and quality of the nurse colonies, the absence and presence of the queen in the rearing colony, grafted larval age, nurse bees age in rearing colonies, period of the queen-less stage, and number of grafted larval cells (Snelgrove, 1949; Ruttner, 1983). In the present study, the highest acceptance rate was observed for one day old grafted larvae (81.11% in each study year) followed by two days old and three days old larvae. Contrarily, Okuyan and Akyol (2018) reported the highest acceptance rate in the season of summer (June and July) for the two days old grafted larvae (85.1%) whereas the lowest for one day old grafted larvae (81.2%). These differences might be due to subspecies differences, and climatic conditions. Rearing seasons have a great influence on the acceptance rate of queen-grafted cells (Koç and Karacaoglu, 2004).

For both commercially effective beekeeping and a good honeybee output, the quality of the queens is essential. A queen's physical attributes, including weight, thorax and head breadth, ovariole count, spermathecal size, and amount of stored spermatozoa, are measured to assess the quality of the queen. High-quality queens tend to have higher performance characteristics than low-quality queens, including resilience to disease, good brood pattern, high grooming and hygienic behavior, low swarming tendency, and high yield of bee products (Hatjina *et al*., 2014; Mattiello *et al*., 2022). Several factors have been reported that strongly influence the queen weight like rearing season, grafted larval age, supplemental feeding and density of bees in starter hive, and genetic variations (Mirza *et al*., 1967; Gencer *et al*., 2000; Emsen *et al*., 2004; Mahbobi *et al*., 2012, 2014). Additionally, previous studies reported that the queens from different diet conditions had significantly different sperm counts in their spermatheca, body size, and weight (Delaney *et al*., 2011; Tarpy *et al*., 2012). In the current study, the higher queen weight was recorded for one day old grafted larvae (159.20 mg in 2020 and 156.90 mg in 2021), whereas the lowest was

for three days old grafted larvae. Several studies have also reported similar findings. Gencer *et al*. (2000) found that the emerged queens from one day old grafted larvae (166.6 mg) were heavier than those that emerged from two-day-old grafted larvae (160.8 mg). Moreover, these heavier queens produced more brood area than lighter queens. But, colonies under nutritional stress such as pollen and amino acids will badly affect brood production. If the queens were produced under nutrient stress conditions, the poor brood pattern is likely the most relevant cause of unhealthy queens.

Moreover, Okuyan and Akyol (2018) reported the highest queen weight (173.59 mg) in one day old grafted larvae, whereas the lowest was in three days old grafted larvae (158.69 mg). Queens reared from one day old grafted larvae were heavier in weight (158.83 mg) with superior quality than queens reared from two and three days old grafted larvae (Mahbobi *et al*., 2012). Previous research found a positive association between the body weight of emerging queens and numerous characteristics of their reproductive organs. These characteristics include the number of ovarioles, the diameter of the spermatheca, and the amount of spermatozoa that are stored (Hatjina *et al*., 2014; Arslan *et al*., 2021).

The age of the grafted larvae greatly influences the morphological characteristics of queen bees; these morphological characteristics play a key role in the reproductive success or fertility of queen bees (Delaney *et al*., 2011). It has been found that the heavy-weight and superior queen bees have greater spermathecae and number of sperm in them as compared to light-weight queens (Akyol *et al*., 2008). In the present study, the queen's morphological characteristics, i.e., the body weight of the queen, head width, head length, thorax width, thorax length, wing width, wing length, and body length, were greatly affected by the age of grafted larvae. The majority of the studies have reported the significant effect of grafted larval age on morphological characteristics mostly on body weight and body length (Gencer *et al*., 2000; Mahbobi *et al*., 2012; Njeru *et al*., 2017; Okuyan and Akyol, 2018; De Souza *et al*., 2019). Queens produced from worker larvae older than one day are morphologically more identical to workers than queens produced from young larvae. Moreover, queens produced from older grafted larvae generally have smaller reproductive structures (Woyke, 1971).

Additional research could be carried out in the future to corroborate the findings of this study by gathering data from queens raised in cups of various diameters and determining the exterior and interior morphological characteristics of bee queens.

CONCLUSIONS

The acceptance rate and morphological characteristics of grafted queen bees in this experiment were significantly affected by the age of grafted larvae. The queens produced from one-day-old grafted larvae are higher in acceptance rate, greater body weight, body length, head length and width, thorax length and width, and wing length and width than the two and three days old larvae. Therefore, grafting one day old larvae is strongly recommended for queen-rearing to rear superior-quality honeybee queens. From a more practical edge perception, these findings can provide guidance in efforts to improve honeybee queen quality in profitable queen rearing to resolve issues underlying the increased status of queen failures in the apiculture of Pakistan. For future research, the possible physiological or hormonal factors that affect queen morphological characteristics must be examined.

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Ethical statement

Not applicable.

Statement of conflict of interest

The authors have declared no conflict of interest.

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