

Feeding response of *Aedes aegypti* and *Anopheles dirus* (Diptera: Culicidae) using out-of-date human blood in a membrane feeding apparatus

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ABSTRACT: The colonization of *Aedes aegypti* and *Anopheles dirus* was performed using out-of-date human blood from a blood bank as a nutritional supply dispensed from a common artificial feeder. Preserved human blood was collected and used for feeding on days 5, 15, and 25 after date of expiration and dispensed from a common artificial feeder to rear the mosquitoes. *Ae. aegypti* had a feeding rate of 78.7, 62, and 18% at the respective intervals while *An. dirus* had a rate of 80, 56.8, and 7.3% on the same respective days. Direct feeding on live hamsters resulted in a rate of 96 and 90% for *Ae. aegypti* and *An. dirus*, respectively. Although egg production rates decreased from the day 5 feeding to the day 25 feeding, all of the developmental stages resulting from *An. dirus* fed at day 5 and 15 showed insignificant differences when compared with direct feeding on the blood of a hamster. *Journal of Vector Ecology* 35 (1): 149-155. 2010.

Keyword Index: *Aedes aegypti*, *Anopheles dirus*, colonization, out-of-date blood, artificial feeding, Thailand.

INTRODUCTION

The laboratory colonization of *Aedes aegypti* and *Anopheles dirus*, the main human vectors for dengue and malaria in Thailand, respectively, is of major importance for purposes of research, control, and experimentation. For most mosquito species, blood-feeding is essential for the production of viable eggs and blood-feeding on laboratory animals has some definite advantages. It has been reported that females of *Aedes* and *Anopheles* mosquitoes maintained on blood from live animals laid a higher number of eggs during their lifetimes and had greater longevity than those fed only sugar (Day et al. 1994, Straif and Beier 1996).

Various species of live animals, including mice, rats, gerbils, guinea pigs, hamsters, rabbits, chickens, non-human primates, and humans, have been used successfully to blood-feed mosquitoes (Nayar and Sauerman 1977, Klein et al. 1986, Canyon et al. 1999, Harrington et al. 2001, Braks et al. 2006, Xu et al. 2006, Xue et al. 2008). However, the use of live animals has many drawbacks, including pain and distress caused by blood feeding and additional/unsustainable expense for the maintenance of animals in the laboratory (Kasap et al. 2003). Moreover, there are evidences of accidental disease transmission and hypersensitivity to mosquito bites (Bailey et al. 1978). Evidently, it is somewhat effective, in terms of cost, safety and welfare of animals, to employ an artificial membrane feeding technique.

An artificial membrane feeding technique has been utilized for at least 40 years. The technique has been

proven to be not only an effective method for delivering a blood meal to mosquitoes but has also been used to determine infection and transmission thresholds of viruses, *Plasmodium* spp., and filarial worms in the mosquitoes (Collins et al. 1964, Rutledge et al. 1964, Ponnudurai et al. 1971, Sattabongkot et al. 2003, Pothikasikorn et al. 2007). Various kinds of membrane have been applied in this technique, for instance, the skin of bat's wings, latex condoms, and paraffin films (Bailey et al. 1978, Wirtz and Rutledge 1980, Hagen and Grunewald 1990, Novak et al. 1991, Kasap et al. 2003, Rampersad and Ammons 2007). As for the blood meal, blood from various hosts, such as avian blood, rodent blood, mammalian blood, and stored human blood from blood banks have been used (Bunner et al. 1989, Nasirian and Ladonni 2006).

An effective storage system for human blood has been sought for the past 90 years (Hess 2006). At the present time, the common RBC storage solutions (anticoagulants) work well for blood banks, allowing three to seven weeks storage. There are many blood storage solutions or anticoagulant formulae in use from the blood banks of Thailand, for instance, acid citrate dextrose (ACD), citrate phosphate dextrose (CPD), (citrate phosphate dextrose plus adenine (CPDA-1), and citrate phosphate dextrose with additive solution (CPD-AS). However, the storage solution ACD is the one commonly used in many blood banks in hospitals throughout Thailand. This solution provides for 21 days of effective preservation of RBC. After day 21, RBC will lose some components and hemolysis may commence

(Sawant et al. 2007). Hence, the standard of 21 days is used to determine the expiration date of preserved human blood from blood banks. Since expired preserved human blood is discarded every day, it would be interesting to determine whether the two important vectors, *Ae. aegypti* and *An. dirus*, can be colonized using out-of-date preserved human blood supplied by a blood bank through an artificial feeding technique. This technique will provide an alternative blood source from the common practice of feeding the mosquitoes on live hamsters for purposes of colonization.

MATERIALS AND METHODS

Mosquitoes

This investigation used material from colonies of *Aedes aegypti* and *Anopheles dirus* that have been maintained under laboratory conditions at Kasetsart University, Bangkok, Thailand. Newly emerged adults were provided cotton pads soaked with 10% sucrose solution. Seven-day-old non-blood-fed *Ae. aegypti* females were used in this study. Due to the same difficulties in handling test specimens of *An. dirus*, only 12-day post-emergence females were used in the experiment. All females were sugar starved for at least 12 h before using (Rutledge et al. 1964).

Feeding apparatus and technique

Out-of-date preserved human blood was obtained from the blood bank of Ramathibodi Hospital, Bangkok, Thailand. The blood was stored in an ACD solution and refrigerated at 4° C until use. The blood was collected on days 5, 15, and 25 after the expiration date from the same blood bag and used as a feeding substrate. Ordinarily, a hamster from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Bangkok, Thailand, was used for blood feeding, strictly following the NLAC-MU guidelines.

Mouse skin was chosen as a membrane for the artificial feeding set-up due to its effectiveness and availability as previously described by Pothikasikorn et al. (2007). Briefly, the carcasses of mature mice obtained from the laboratory of the Microbiology and Biology Departments at the Faculty of Science, Mahidol University were skinned, the fur clipped, and all fat and connective tissues scraped or pulled off. The mouse skin was then frozen until use (Pothikasikorn et al. 2007).

The feeder apparatus was made of heat-resistant glass to which was attached a cylindrical water-jacket to contain the blood meal. The feeding component was set up and kept warm through the entry of tepid water from a lower inlet and its exit from an upper outlet connected to the water bath (Rutledge et al. 1964). The bottom of the cylinder had a circular lip over which the membrane could be securely tied with a rubber band to close the base of the feeder. Five ml of blood meal was placed inside the feeder. Plasticine was used to close the top of the tube of the chamber. Prior to feeding, the blood was warmed and maintained between 32–37° C in the cylinder of the feeder apparatus.

Feeding method

For each set of outdated preserved human blood, 150 females of each mosquito species were divided into three groups of 50 each. Each group was placed in a plastic bowl cage (6 cm high and 12 cm in diameter) covered with nylon mesh. Three feeders, each fitted with a mouse-skin membrane, were set up in tandem so that each one could be placed on top of a separate mosquito holding cage. Feeding tests were conducted on the mosquitoes from each cage with each session lasting for 30 min for *Ae. aegypti* and 60 min for *An. dirus* (Pothikasikorn et al. 2007). While mosquitoes fed, the artificial blood meal was periodically shaken to prevent precipitation of blood cells. The feeder temperature ranged from 32° to 37° C (mean 34.5° C) while the ambient temperature range was 23°–27° C (mean 25° C) and the relative humidity was 80%. Direct feeding on the hamster was done by placing the hamster in the 30 cm³ stainless steel cage with batches of 50 female mosquitoes fed for 30–60 min. Due to ethical reasons and complicated processes, fresh human blood could not be obtained. Instead, the mosquitoes were fed on a hamster as controls.

Determination of developmental rates

After being fed, the mosquitoes were removed and examined to determine the feeding rate. The surviving female mosquitoes were transferred to the 30 cm³ stainless steel cages and provided with 10% sucrose solution.

Three to four days after females were fully engorged, *An. dirus* deposited eggs on a 3 x 15 cm piece of filter paper lining a labeled 150 ml beaker containing 75 ml of clean water. Three to four days after feeding, *Ae. aegypti* deposited their eggs on a 2.5 cm diameter piece of absorbent paper placed on cotton soaked with clean water inside a small plastic bowl (2.0 cm diameter and 1.5 cm high), and the number of eggs of both mosquito species were recorded to determine the oviposition rate.

One to two days after oviposition, the papers with eggs were removed from the cages and placed in a 22x35x7 cm pan half-filled with clean water and the number of larvae was recorded to determine the hatching rate.

Larvae were reared to the pupal stage in a 22x35x7 cm pan filled half-way with clean water and fed daily with ground rabbit chow for nutrition. The rate of pupation and adult formation were recorded respectively. Adults were provided a 10% sucrose solution on-soaked cotton pads.

We subsequently compared the direct feeding on a hamster method and the expired human blood feeding method with each of the developmental rates using a one-way ANOVA – LSD throughout the study. Analysis was performed using the Statistix 9.0 Analytical Software (Tallahassee, FL, U.S.A.).

RESULTS

Ae. aegypti and *An. dirus* were reared using out-of-date human blood from a blood bank in an artificial feeding apparatus using a mouse skin as the membrane, collected on days 5, 15, and 25 after its expiration date. The feeding

Table 1. Feeding rate of *Ae. aegypti* and *An. dirus*, comparing direct feeding on hamsters to feeding on out-of-date preserved human blood. Percentages in each row followed by the same letter are not significantly different ($P \leq 0.05$) by one-way ANOVA - LSD.

Mosquito species	No. engorged females / total (%)			
	Direct feeding on live hamster	Human blood 5 d post-expiration	Human blood 15 d post-expiration	Human blood 25 d post-expiration
<i>Aedes aegypti</i>	144/150 (96.0) ^a	118/150 (78.7) ^b	93/150 (62.0) ^c	27/150 (18.0) ^d
<i>Anopheles dirus</i>	135/150 (90.0) ^a	120/150 (80.0) ^b	85/150 (56.7) ^c	11/150 (7.3) ^d

Table 2. Effect of feeding responses for outdated preserved human blood on the oviposition rate, hatching rate, and pupation rate to adult formation rates of *Ae. aegypti* and *An. dirus* in comparison to direct feeding on a hamster. Percentages in each column followed by the same letter are not significantly different ($P \leq 0.05$) by one-way ANOVA - LSD.

Control / outdated blood	No. eggs/engorged females (mean $\pm$ SD)		Sum of larvae (mean % hatching rate $\pm$ SD)		Sum of pupae (mean % pupation rate $\pm$ SD)		No. of adults (mean % adult formation rate $\pm$ SD)	
	<i>Ae. aegypti</i>	<i>An. dirus</i>	<i>Ae. aegypti</i>	<i>An. dirus</i>	<i>Ae. aegypti</i>	<i>An. dirus</i>	<i>Ae. aegypti</i>	<i>An. dirus</i>
Live hamster	2,211/144 (15.3 $\pm$ 1.1) ^a	2,400/135 (17.9 $\pm$ 1.1) ^a	1,419 (64.6 $\pm$ 6.2) ^a	1,172 (48.8 $\pm$ 0.1) ^a	654 (46.1 $\pm$ 2.6) ^a	630 (53.7 $\pm$ 3.2) ^a	417 (63.8 $\pm$ 0.6) ^a	488 (77.5 $\pm$ 0.7) ^a
human blood 5 d post-expiration	1,504/118 (12.7 $\pm$ 0.2) ^b	1,870/120 (15.6 $\pm$ 0.1) ^a	793 (52.7 $\pm$ 0.8) ^b	877 (46.9 $\pm$ 2.1) ^a	463 (58.3 $\pm$ 2.3) ^b	460 (52.5 $\pm$ 1.2) ^a	272 (58.6 $\pm$ 2.6) ^a	387 (84.1 $\pm$ 1.0) ^a
human blood 15 d post-expiration	932/93 (10.0 $\pm$ 0.0) ^c	745/85 (8.8 $\pm$ 1.5) ^b	484 (52.0 $\pm$ 1.0) ^b	345 (46.6 $\pm$ 2.6) ^a	266 (55.3 $\pm$ 6.0) ^b	180 (52.1 $\pm$ 1.1) ^a	167 (62.9 $\pm$ 7.9) ^a	150 (83.3 $\pm$ 0.0) ^a
human blood 25 d post-expiration	240/27 (8.8 $\pm$ 0.9) ^c	51/11 (2.8 $\pm$ 4.9) ^c	113 (47.9 $\pm$ 3.7) ^b	22 (43.1 $\pm$ 24.9) ^b	62 (54.3 $\pm$ 3.5) ^b	8 (12.1 $\pm$ 21.0) ^b	28 (43.1 $\pm$ 9.3) ^b	6 (25.0 $\pm$ 43.3) ^b

rates of *Ae. aegypti* and *An. dirus* significantly ($P \leq 0.05$) decreased as the out-of-date blood became older (Table 1 and Figure 1). For example, the feeding rate of *Ae. aegypti* on blood collected on day 5 after its expiration date was approximately four times greater than on blood collected on day 25 after its expiration date. Similarly, the feeding rate

of *An. dirus* on blood collected on day 5 after its expiration date was approximately eleven times greater than on blood collected on day 25 after its expiration date (Table 1). Additionally, the rates of feeding on various ages of out-of-date blood were significantly lower than the rates of feeding on live animal controls for both mosquito species ($P \leq 0.05$).

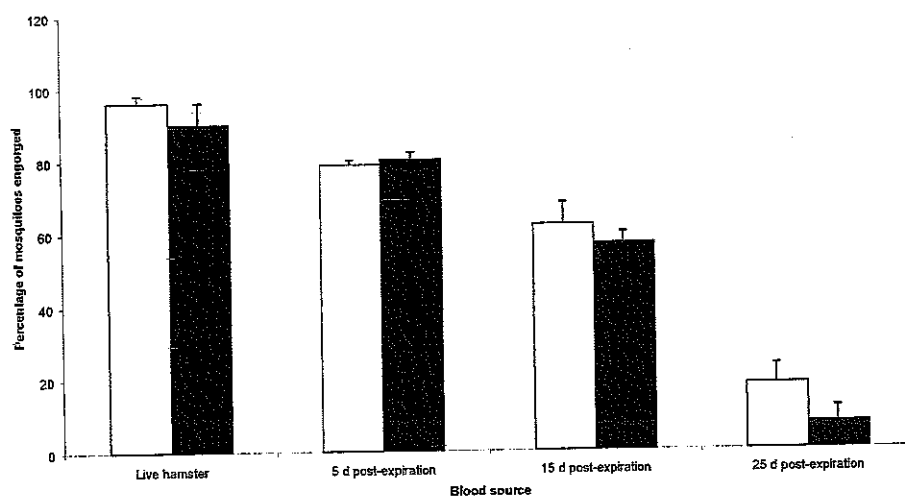


Figure 1. Effect of blood source on feeding rate of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

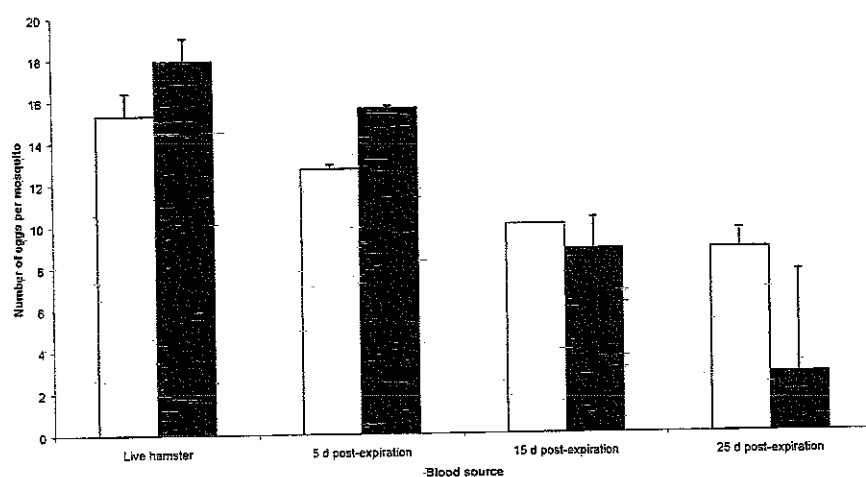


Figure 2. Effects of blood source on egg production of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

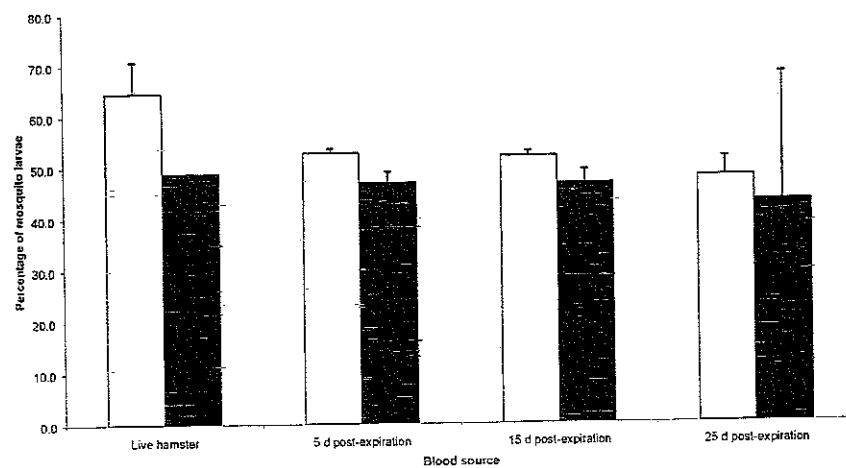


Figure 3. Effects of blood source on egg hatching for *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

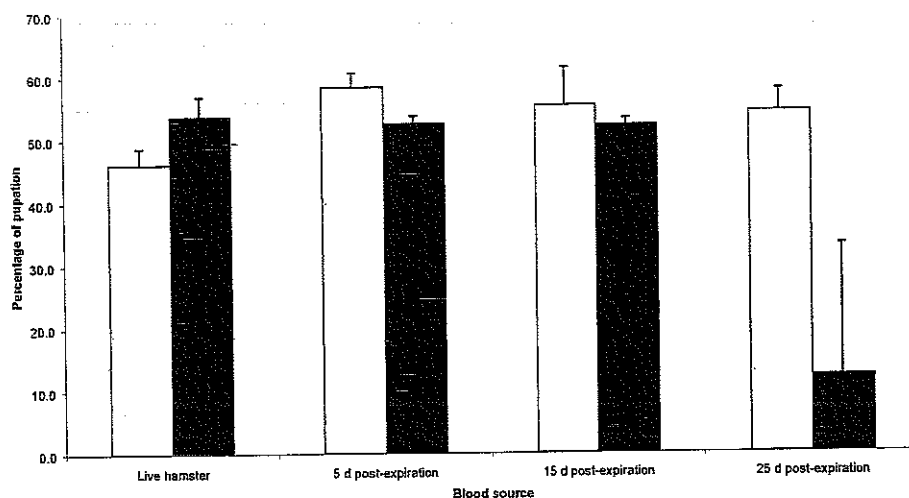


Figure 4. Effects of blood source on the pupation of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

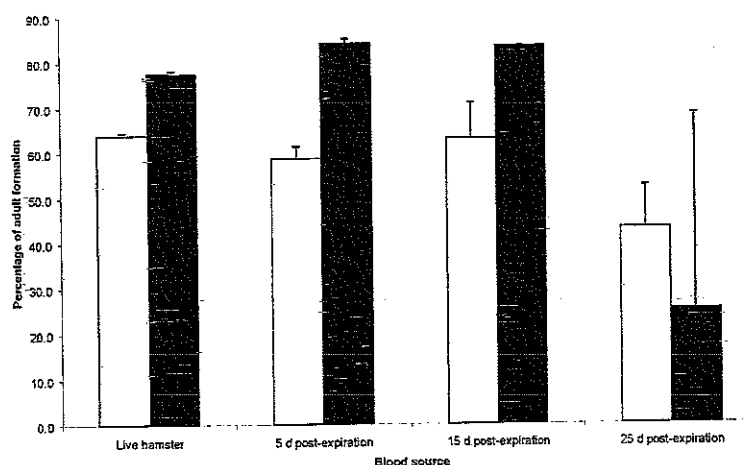


Figure 5. Effects of blood source on adult development of *Ae. aegypti* (open bars) and *An. dirus* (solid bars).

Feeding rates on live hamsters were 0.96 for *Ae. aegypti* and 0.90 for *An. dirus*, whereas rates were comparatively low on blood collected on day 25 after its expiration date for both species (0.18 for *Ae. aegypti* and 0.07 for *An. dirus*) (Table 1). No mortality was observed in test populations of both species reared under laboratory conditions.

Age of out-of-date blood appears to affect the fecundity rates of both species of mosquitoes as shown in Table 2 and Figure 2-5. The egg production rate of *Ae. aegypti* mosquitoes steadily decreased when fed on out-of-date human blood collected on day 5, 15, and 25 after expiration date, ranging from 12.7, 10.0, to 8.8 eggs per female, respectively, compared to 15.3 eggs per female in the control group (Table 2). The rates of hatching from egg to 1st instar larva of *Ae. aegypti*, when fed on days 5, 15, and 25 after blood expiration, still showed significant decreases when compared with the control ($P \leq 0.05$). In contrast, pupation

rates (1st instar larva developing to pupa) were significantly higher in out-of-date blood fed groups than the control group ($P \leq 0.05$). While the group that fed on the most aged out-of-date blood (25 d after expiration date) showed a significant reduction in the adult development rate, there was no significant difference in such rate among mosquitoes fed on the more recently out-of-date blood compared to the control group ($P \leq 0.05$).

DISCUSSION

Although blood meals from live animals such as hamsters and guinea pigs have been used routinely in maintaining mosquito colonies in laboratories, their drawbacks include ethical issues and often unsustainable costs. This study has shown that recently expired stored human blood, normally discarded by blood banks, is an

alternative to live animal feeding.

The decrease in the feeding success on out-of-date blood of both mosquitoes shown in Figure 1 may be because the expired blood is less attractive as a food source to mosquitoes. It has been reported that stored RBCs become fractionated as they age, thus depleting essential substances such as adenine nucleotides, potassium, diphosphoglycerate (DPG), ATP stores, amino acids, and lipid (Hosoi 1959, Galun et al. 1984, Tinmouth and Chin-Yee 2001, Greenwalt et al. 1991, Hess 2006, Muller-Steinhardt et al. 1997, Gammon et al. 2000, Sawant et al. 2007, Hurd et al. 1995, Dimond et al. 1956, Briegel 1990, Scott et al. 1993, Harrington et al. 2001). Although the more recent out-of-date blood did not affect the fecundity of *An. dirus*, the decreases in nutritional components in aged out-of-date blood seemed to significantly decrease the fecundity rate of both species compared to the control group. This is probably due to the qualitative differences in host blood (hamster vs human blood) which would affect mosquito egg production (Nayar and Sauerman 1977). Spielman and Wong (1974) reported that the amino acid isoleucine was found in lower titers in human blood compared with other vertebrate species such as chicken and mice, and suggested that this might be a limiting factor in mosquito egg production. As confirmed by Harrington et al. (2001), *Ae. aegypti* may thus benefit from an accumulation of more energy reserves and a fitness advantage when they ingest human blood (low isoleucine) rather than rodent or chicken blood (high isoleucine).

Although the recent out-of-date bloods (5 and 15 d after expiration dates) seemed to reduce the hatching rates of *Ae. aegypti* mosquitoes (Figure 3), the rate of adult development was not affected by the out-of-date blood (Figure 5). More interestingly, the rate of pupation of this mosquito species fed on the recent out-of-date blood was significantly increased compared to the control group (Figure 4). As for *An. dirus* mosquitoes, the recent out-of-date bloods (5 and 15 d after expiration dates) did not appear to significantly affect rates of hatching, pupation, or adult development (Figures 3, 4, 5). A possible explanation may be related to the nutrition of female mosquitoes. Blood and sugar are essential for survival and reproduction in female mosquitoes (Briegel and Horler 1993, Foster 1995), and the recent out-of-date human blood could provide essential components required for reproduction and development.

In summary, it was found that all of the surviving female mosquitoes fed preserved human blood outdated by between 5 and 15 d displayed only slight differences in fecundity when compared to the controls. We therefore suggest that preserved human blood which is out-of-date by up to 10 d could be used as a source of nutrition to rear *Ae. aegypti* and *An. dirus* mosquitoes as an alternative to directly feeding on a hamster. These experiments show that the freshness of the blood showed no advantage over outdated preserved human blood samples provided that cell lysis had not occurred in the RBC during collection and storage.

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