Breeder Diet Strategies for Generating *Ttpa*-Null and Wild-Type Mice with Low Vitamin E Status to Assess Neurological Outcomes

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ABSTRACT

Studying vitamin E [α-tocopherol (α-T)] metabolism and function in the brain and other tissues requires an animal model with low α-T status, such as the transgenic α-T transfer protein (*Ttpa*)–null ($Ttpa^{-/-}$) mouse model. $Ttpa^{+/-}$ dams can be used to produce $Ttpa^{-/-}$ and $Ttpa^{+/+}$mice for these studies. However, the α-T content in $Ttpa^{+/-}$ dams' diet requires optimization; diets must provide sufficient α-T for reproduction, while minimizing the transfer of α-T to the offspring destined for future studies that require low baseline α-T status. The goal of this work was to assess the effectiveness and feasibility of 2 breeding diet strategies on reproduction outcomes and offspring brain α-T concentrations. These findings will help standardize the breeding methodology used to generate the $Ttpa^{-/-}$ mice for neurological studies. *Curr Dev Nutr* 2020;4:nzaa155.

Keywords: vitamin E, reproduction, breeding diet strategies, α-tocopherol transfer protein, mouse

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Abbreviations used: AVED, ataxia with vitamin E deficiency; CON, control (diet); E-DOSE, control diet followed by vitamin E–deficient diet; LOW-E, low vitamin E diet; *Ttpa*, α-tocopherol transfer protein; VED, vitamin E–deficient diet; α-T, α-tocopherol; α-TA, α-tocopheryl acetate; α-TTP, α-tocopherol transfer protein.

Introduction

Vitamin E [α-tocopherol (α-T)] is essential for animal reproduction and embryogenesis (1, 2). Female rodents, in particular, require α-T for normal placental development (3, 4), and fetal resorption in α-T–deficient breeders is well established. Accordingly, the rat fetal-resorption assay was widely used to measure the biological activity of vitamin E (5).

Human reproduction may also require α-T, but the most pronounced symptoms of human α-T deficiency are neurological (6). Nervous system–related complications are also seen in other animals, such as rodents (7), zebrafish (2), chicks (8), horses (9), and monkeys (10). However, animals generally require long-term dietary α-T restriction to deplete body stores, and the brain is especially resistant to α-T depletion (11, 12). While slow tissue α-T depletion is beneficial from a biological perspective, it is a hurdle for choosing an appropriate animal model for vitamin E studies. Long-term dietary α-T restriction is also not possible when studying nervous system development in young animals.

The transgenic α-T transfer protein (*Ttpa*)–null ($Ttpa^{-/-}$) mouse model offers a compelling alternative to other animal models. The $Ttpa^{-/-}$ mouse was developed via targeted disruption of α-T transfer protein (α-TTP), which is the key regulatory protein for α-T (3, 13). Hepatic α-TTP facilitates the transfer of α-T into very-low-density lipoproteins, which then circulate in the body and distribute α-T to extrahepatic tissues. Without this protein, the majority of α-T is catabolized in the liver and does not reach extrahepatic tissues like the brain, resulting in low α-T stores (14).

Humans with rare functional mutations in the α-TTP gene also have disrupted α-T tissue deposition and low α-T status. Consequently, they develop the condition called ataxia with vitamin E deficiency (AVED), which is characterized by deficits in motor coordination and peripheral neuropathy (6). Because adult $Ttpa^{-/-}$ mice develop a similar neurological phenotype as humans with AVED , this model has proven useful for assessing the molecular and behavioral consequences of vitamin E deficiency, as well as possible treatment interventions (15).

Placental transfer of α-T is known to occur in humans (16) and other animals such as guinea pigs (17), but the mammary gland may be the primary route of α-T transmission to offspring (18–20). Therefore, α-T–depleted milk is required to minimize tissue accumulation of α-T in pups. This can be achieved by feeding $Ttpa^{-/-}$ dams a vitamin E–deficient diet (VED) and then cross-fostering $Ttpa^{+/+}$ pups with these $Ttpa^{-/-}$ dams (Justin Rhodes, Chron-Si Lai, Matthew Kuchan, Jonathan Mun, Kristy Du, unpublished data, 2017). However, implementing this cross-fostering protocol is very complex.

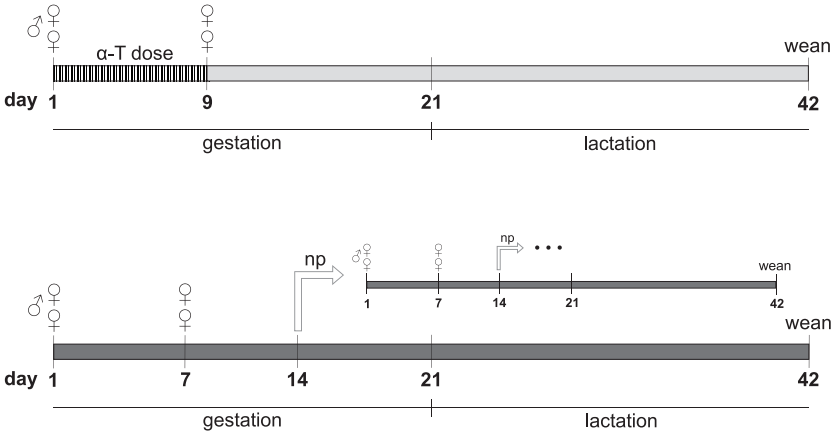

FIGURE 1 E-DOSE (A) and LOW-E (B) breeding diet strategies used to generate *Ttpa*$^{+/+}$ and *Ttpa*$^{-/-}$ weanlings with low vitamin E status. *Ttpa*$^{+/-}$ dams used for E-DOSE were fed an α-T "dose" (AIN-93G diet, 75 mg α-TA/kg), followed by a vitamin E–deficient diet (below α-TA limit of detection, 0.49 mg α-TA/kg). LOW-E dams were continuously fed a low vitamin E diet (35 mg α-TA/kg). Male *Ttpa*$^{+/-}$ breeders were removed from mating cages after either 9 d (E-DOSE) or 7 d (LOW-E). If identified as not pregnant, LOW-E dams were mated again, but E-DOSE dams were not rebred until the next 42-d cycle. E-DOSE, control diet followed by vitamin E–deficient diet; LOW-E, low vitamin E diet; np, not pregnant; *Ttpa*, α-tocopherol transfer protein; α-TA, α-tocopheryl acetate; ♀, female; ♂, male.

In addition to generating simultaneous pregnancies, *Ttpa*$^{-/-}$ dams require high dietary levels of α-T (~1000 IU/kg diet) for fertility (13). Therefore, *Ttpa*$^{-/-}$ dams have to be switched to a VED ~7 d before cross-fostering *Ttpa*$^{+/+}$ pups (Rhodes, Lai, Kuchan, Mun, Du, unpublished data). This approach is labor intensive and not feasible for many studies, especially those requiring large numbers of animals.

A more practical approach is to use *Ttpa*$^{+/+}$ and *Ttpa*$^{-/-}$ littermates generated from *Ttpa*$^{+/-}$ mouse crosses. This has been the chief strategy since the *Ttpa*$^{-/-}$ mouse model was developed. *Ttpa*$^{+/-}$ females do not require high dietary levels of α-T like *Ttpa*$^{-/-}$ females. However, the specifications of the *Ttpa*$^{+/-}$ breeder diet warrant attention. What is the best way to provide dams with sufficient dietary α-T for reproduction, while minimizing the transfer of α-T to offspring? What methodology allows for consistency between animal cohorts and between laboratories?

We evaluated 2 *Ttpa*$^{+/-}$ breeding diet strategies that can be used for future studies, particularly those focused on neurological endpoints. We initially implemented these strategies to generate *Ttpa*$^{-/-}$ mice for our own nervous system–focused studies. However, while the analyses presented here are secondary in nature, they could provide valuable guidance for researchers using this mouse model. The diet strategies we tested included an α-T "dose" approach (E-DOSE) and a consistent low dietary vitamin E concentration approach (LOW-E) (**Figure 1**). For the E-DOSE strategy, dams were given a standard control diet containing vitamin E during the first 9 d of gestation, followed by a VED until weaning. This strategy was inspired by previous studies using wild-type mouse strains to test fertility, in which dams received α-T doses at the beginning of gestation (18, 21). The LOW-E strategy is simpler to conduct, as dams are provided a stable low vitamin E–containing diet throughout gestation and lactation. Similar approaches for female *Ttpa*$^{+/-}$ breeders have been used in recent studies (22).

Our objective was to compare the effectiveness of E-DOSE versus LOW-E on pregnancy and offspring outcomes. We also discuss their feasibility and recommend possible research applications for each breeder diet strategy.

Methods

Animals

All animal procedures followed protocols approved by the University of Illinois Institutional Animal Care and Use Committee. Mice were housed in shoebox cages and maintained in environmentally controlled conditions (12:12-h light-dark cycle, 22°C, 60% humidity). *Ttpa*$^{-/-}$ mice (B6.129S4-Ttpatm1Far/J) were obtained from Jackson Laboratory (JAX stock #003823). *Ttpa*$^{-/-}$ males were crossed with C57BL/6J (*Ttpa*$^{+/+}$) females (F0 generation) to produce heterozygous *Ttpa*$^{+/-}$ offspring (F1 generation). F0 crosses included *Ttpa*$^{-/-}$ males because, unlike *Ttpa*$^{-/-}$ females, they do not need high dietary α-T concentrations for fertility (13). Therefore, F0 male and female breeders were fed an AIN-93G diet [75 mg all-*rac*-α-tocopheryl acetate (α-TA)/kg diet], and custom diet formulations were not necessary during this stage of breeding.

F1 animals (*Ttpa*$^{+/-}$) were used for the diet strategies described herein and for generating F2 animals (*Ttpa*$^{+/+}$ and *Ttpa*$^{-/-}$). F1 females assigned as breeders were weaned onto either a VED (E-DOSE diet strategy; <0.49 mg α-TA/kg diet) or a diet containing low levels of vitamin E (LOW-E diet strategy; 35 mg all-*rac*-α-TA/kg diet). They were fed these diets until breeding (≥6 wk of age). The α-TA concentration for LOW-E is very similar to the estimated vitamin E requirement for mice (32 mg all-*rac*-α-TA/kg diet) (23). Of note is that the AIN-93G diet has ~2 times this concentration, due to the increased level of PUFAs in the 93G diet compared to the older AIN-76G formulation (24).

Tail snips from F2 animals were genotyped using specific primers for *Ttpa* as previously described (13). To analyze brain α-T content, male and female $Ttpa^{+/+}$ ($n = 19$) and $Ttpa^{-/-}$ ($n = 25$) mice were killed at weaning age (postnatal day 21) via carbon dioxide asphyxiation followed by cervical dislocation. Brains were excised and weighed, flash-frozen in liquid nitrogen, and stored at −80°C until analysis.

No power analysis was conducted because evaluating breeding performance was not our primary objective, and performance was unknown before we began the work. This report was compiled as a secondary analysis. We began breeding once we had 6 $Ttpa^{+/-}$ dams because this seemed like a sufficient starting place for generating our study animals. We continually added breeders as we produced more from the F0 crosses.

Diets

AIN-93G (75 mg all-*rac*-α-TA/kg diet) and modified-AIN-93G formulations were used for these experiments (Research Diets, Inc.). The base formulation for modified diets has been previously described (25). The primary modification was the use of hydrogenated coconut oil as the predominant lipid source, as it is naturally low in vitamin E. Unfortunately, vitamin E–stripped corn and soybean oils were not available for this study. Sufficient levels of essential fatty acids for growth (23) were achieved by adding a minimal amount of soybean oil.

The modified base formulation served as the VED diet (below the α-TA limit of detection, <0.49 mg α-TA/kg diet) for the E-DOSE diet strategy; all-*rac*-α-TA (Sigma-Aldrich) was added to the base formulation to prepare the LOW-E diet (35 mg all-*rac*-α-TA/kg diet).

Control diet strategy

The control diet (CON) strategy was used for preliminary experiments to test the transfer of α-T to $Ttpa^{+/+}$ and $Ttpa^{-/-}$ weanlings' brains. These analyses justified the use of α-T–restrictive diet strategies (E-DOSE and LOW-E) to reduce offspring brain α-T concentrations.

A total of 6 $Ttpa^{+/-}$ females were bred with $Ttpa^{+/-}$ males and fed AIN-93G ad libitum throughout gestation and lactation. The breeding configurations included both trios (2 females, 1 male) and standard pairings (1 female, 1 male), and males were removed from mating cages after 7 d. The body mass of each $Ttpa^{+/-}$ female was recorded weekly to track pregnancy status. If a dam's body mass did not increase >3 g by the third measurement (2 wk after pairing), the dam was considered not pregnant and was rebred. A breeding cycle for the CON strategy was defined as the time between pairing and rebreeding, so the length of the breeding cycles ranged from 2 to 6 wk depending on whether or not the dam became pregnant.

E-DOSE diet strategy

For the α-T "dose" approach (E-DOSE), we implemented a 42-d breeding cycle, based on 21-d gestation and lactation periods (Figure 1). The goal of this diet strategy was to provide sufficient dietary α-T during placental development, when there is limited α-T transmission to the fetus, while minimizing α-T transfer from milk (4, 18, 21, 26).

A trio mating format was used for breeding (2 $Ttpa^{+/-}$ females, 1 $Ttpa^{+/-}$ male). Breeders were fed AIN-93G ad libitum for the first 9 d as the α-T "dose." On day 9, sires were removed from the breeder cages, and the dams' diet was switched to the VED diet for the remainder of gestation and lactation. To monitor pregnancy status, we recorded the body mass of each dam on a weekly basis. Dams were considered pregnant when there was a >3 g increase in body mass. Regardless of whether or not dams became pregnant, they were not rebred until the next 42-d cycle, for consistency between cohorts.

Dams were housed together during the full breeding cycle, which allowed pups to nurse from both females. This strategy, known as "aunting phenomenon" (27), appeared to increase the frequency of viable litters. Notably, when dams in a single cage had litters on the same day, we could not always determine which dam delivered which offspring. Because our primary goal was to generate F2 progeny for our future study (25), we prioritized maximizing the yield of offspring over distinguishing each dam's litter.

The number of dams bred each week ranged from 6 to 20. Initially, we bred only a few trios each week, but this strategy produced very few viable litters. Therefore, we began increasing the number of dams bred each week to hasten offspring production. Dams varied in age, as newly matured $Ttpa^{+/-}$ dams were added to the breeding colony throughout the 39-wk period, and dams were used for 1–5 breeding cycles.

The E-DOSE diet strategy was developed for our previous study assessing the effects of natural versus synthetic α-T in brains of male 7-wk-old $Ttpa^{-/-}$ mice (25). Generating the relatively low number of male $Ttpa^{-/-}$ mice used for this study ($n = 28$) required ∼7 mo, and these mice were the offspring of only 22 of the 130 total dams bred during this period. With this scheme, we added ∼5 mice onto the study per month.

LOW-E diet strategy

The same trio mating format and dual-female housing approaches were used for the LOW-E diet strategy (Figure 1) to maximize the production of viable offspring. Breeders were fed the LOW-E diet ad libitum throughout gestation and lactation, and males were removed from the mating cages after 7 d. The body mass of each $Ttpa^{+/-}$ female was recorded weekly to track pregnancy. Similar to the CON strategy protocol, if a dam's body mass did not increase >3 g by the third measurement (2 wk after pairing), the dam was considered not pregnant and was rebred. A breeding cycle for the LOW-E strategy was defined as the time between pairing and rebreeding, so the length of the breeding cycles ranged from 2 to 6 wk depending on whether or not the dam became pregnant.

As with the E-DOSE diet approach, the number of LOW-E dams bred each week also varied (8–30 dams). After a dam's first breeding cycle, she was bred up to 6 additional times, and newly matured $Ttpa^{+/-}$ dams were added to the breeding colony throughout the 31 wk.

Pregnancy, gestational, and viability outcomes

Pregnancy, gestational, and viability indexes were used to compare the effect of diet strategies on breeding effectiveness. The formulas used were as follows—pregnancy index: total number of pregnancies/total number of breeding cycles; gestational index: total number of live litters/total number of pregnancies; viability index: total number of litters alive at postnatal day 21/total number of live litters. These formulas were based on those used by Tyl et al. (28) and Ziv-Gal et al. (29) but were modified because we did not collect some of the data used in their formulas (e.g., presence of vaginal plugs). As mentioned above, an increase in body mass of >3 g was considered a pregnancy. The E-DOSE breed-

TABLE 1 Breeding outcomes for E-DOSE and LOW-E diet strategies[1]

Breeding outcome	Breeder diet strategy	
	E-DOSE	LOW-E
Total no. of dams	130	106
Total no. of pregnancies (estimated no. per week)[2]	167 (4.3)	296 (9.4)
No. of offspring[3]	384	928
Average litter size[4]	3.8 ± 0.3*	4.9 ± 0.2

[1] *$P = 0.003$, Mann-Whitney test. E-DOSE, AIN-93G followed by vitamin E–deficient diet; LOW-E, low vitamin E diet.
[2] Total number of mated dams/total number of breeding weeks (E-DOSE: 39; LOW-E: 31).
[3] Values are underestimates. Some pups were cannibalized or found dead/removed from cages and consequently not recorded.
[4] Calculations exclude mice that could not be assigned to a single dam.

ing cycle was always 42 d, while the LOW-E and CON breeding cycle lengths depended on pregnancy status.

When it was not clear which litter or F1 dam the F2 animals belonged to, these offspring were omitted from the gestational index, viability index, and average litter size calculations. This was 1 consequence of housing 2 pregnant females together in 1 cage. A substantial number of offspring for both the E-DOSE diet strategy (151 pups, 14 cages) and the LOW-E diet strategy (178 pups, 31 cages of litters) could not be assigned to a dam. This may have biased our results, particularly for the viability index values. Additionally, multiple mice were found dead and removed from the cage by animal care staff before litter sizes could be recorded.

Brain α-T and diet α-TA analysis

α-T was extracted from ∼100 mg homogenized brain tissue of *Ttpa*$^{+/+}$ and *Ttpa*$^{-/-}$ weanlings. Brain α-T and diet α-TA concentrations were analyzed via HPLC with photodiode array detection as previously described (25, 30).

Statistical analysis

Data were analyzed using GraphPad Prism version 8.1.2 for Windows (GraphPad Software). Shapiro-Wilk and Brown-Forsythe tests were used to evaluate normality and homogeneity of variance, respectively. When data did not pass the normality test even after applying several transformations, nonparametric tests were used. The Mann-Whitney test was used to compare the average litter sizes between E-DOSE and LOW-E dams. The Kolmogorov-Smirnov test was used to compare the distributions of the number of litters delivered by E-DOSE and LOW-E dams. Kruskal-Wallis and Dunn's post hoc tests were used to assess the effect of diet strategy on brain α-T concentrations for each genotype. Differences were considered significant when $P < 0.05$.

Results

Pregnancy and gestational outcomes

The E-DOSE diet strategy yielded 384 recorded pups from 167 pregnancies (using 130 dams) over the 39-wk period, while the LOW-E diet strategy yielded 928 recorded pups from 296 pregnancies (using 106 dams) over 31 wk (**Table 1**). Consequently, the estimated number of pregnancies per week was lower for E-DOSE (∼4) than LOW-E (∼9). The pregnancy index did not differ between the E-DOSE (64%) and LOW-E (64%) diet strategies, but both values were lower than for dams fed the CON diet throughout gestation and lactation (89%) (**Figure 2**). A similar trend was observed for the gestational index, whereby E-DOSE (71%) and LOW-E (70%) were similar but both were less than CON (94%). The majority of E-DOSE dams produced 0 litters (36.2%), 1 litter (43.8%), or 2 litters (13.8%) during the 39 wk (**Figure 3**). There was an increased number of litters delivered by LOW-E dams, which significantly shifted the distribution to the right ($P < 0.001$). A higher percentage of LOW-E dams delivered 2 litters (39.6%), 3 litters (26.4%), 4 litters (10.4%), or 5–6 litters (9.43% combined) during the 31 wk.

Testicular degeneration has been reported in male rats with low α-T status (31) but not in male mice (3, 21). We did not observe any obvious fertility issues in our male *Ttpa*$^{+/-}$ breeders, although this cannot be completely ruled out because we did not assess these outcomes formally. However, these mice were fed an AIN-93G diet when not in the mating cages, so their α-T status likely remained adequate throughout the breeding period.

Offspring outcomes

We recorded the birth of 384 pups produced via the E-DOSE strategy and 928 pups via the LOW-E strategy (Table 1). However, for both diet strategies, an unknown number of offspring were cannibalized or found dead and removed from the cage before being recorded. The average litter size for known offspring from the E-DOSE dams (3.8 ± 0.3) was

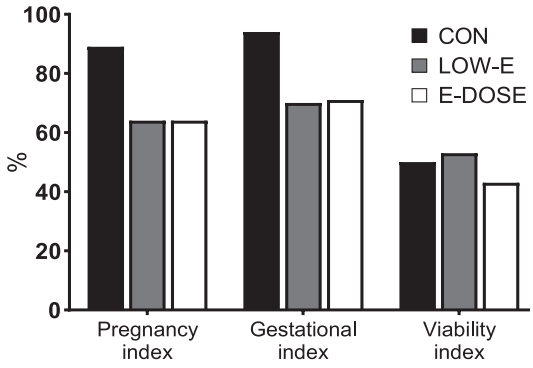

FIGURE 2 Pregnancy, gestational, and viability indexes for *Ttpa*$^{+/-}$ dams bred using different diet strategies. See Methods for definitions of indexes. CON, control (AIN-93G diet); E-DOSE, AIN-93G followed by vitamin E–deficient diet; LOW-E, low vitamin E diet; *Ttpa*, transgenic α-tocopherol transfer protein.

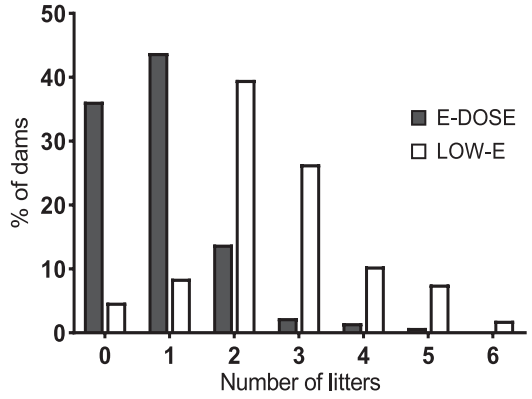

FIGURE 3 *Ttpa*$^{+/-}$ dams bred using the LOW-E strategy produced more litters over the total breeding period than E-DOSE dams ($P < 0.001$, Kolmogorov-Smirnov test). E-DOSE, AIN-93G followed by vitamin E–deficient diet; LOW-E, low vitamin E diet; *Ttpa*, transgenic α-tocopherol transfer protein.

significantly lower than from the LOW-E dams (4.9 ± 0.2) ($P = 0.003$). Both were lower than the estimated 7.0-pup litter size for the wild-type C57BL/6 mouse strain (32). The viability index values were similar between E-DOSE (43%), LOW-E (53%), and CON (50%) dams (Figure 2). This may reflect multiple factors, such as the reduced reproductive success of all *Ttpa*$^{+/-}$ dams and the unknown information from indistinguishable litters (when 2 dams had pups in the same cage).

Ideally, *Ttpa*$^{+/+}$and *Ttpa*$^{-/-}$ mice used for studying vitamin E in the brain have low α-T concentrations at study baseline, which is often weaning age. Dams fed the E-DOSE diet protocol produced *Ttpa*$^{+/+}$and *Ttpa*$^{-/-}$ weanlings with significantly lower brain α-T concentrations than weanlings of dams fed the CON diet (**Figure 4**). LOW-E diet weanlings had numerically lower brain α-T concentrations than CON diet weanlings.

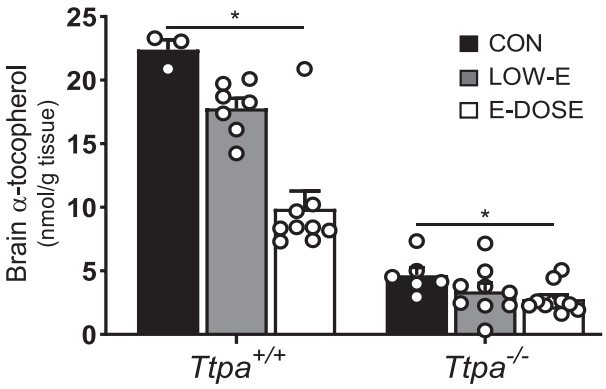

FIGURE 4 Brain α-tocopherol concentrations in homogenized brains of 3-wk-old *Ttpa*$^{+/+}$ and *Ttpa*$^{-/-}$ weanlings. Female *Ttpa*$^{+/-}$ breeders were fed using CON, E-DOSE, and LOW-E diet strategies. Data are expressed as means ± SEMs; $n = 3$–10/group. $^{*}P < 0.05$ by Kruskal-Wallis and Dunn's post hoc tests. Each circle represents an individual mouse's measured value. Lower limit of detection: 0.12 nmol α-tocopherol/g. CON, control (AIN-93G diet); E-DOSE, AIN-93G followed by vitamin E–deficient diet; LOW-E, low vitamin E diet; *Ttpa*, transgenic α-tocopherol transfer protein.

TABLE 2 Recommendations for using E-DOSE and LOW-E diet strategies[1]

Study consideration	Breeder diet strategy	
	E-DOSE	LOW-E
Low α-T status (↓ transfer to offspring)	x	
Minimize resources (diets, time, dams)		x
Animal stage of life		
Young (development)	x	
Adult (aging)		x
Duration of feeding intervention		
Short-term	x	
Long-term		x

[1]E-DOSE, AIN-93G followed by vitamin E–deficient diet; LOW-E, low vitamin E diet throughout breeding; α-T, α-tocopherol; ↓, reduced.

Mendelian genetics predict that 25% of the offspring will be the *Ttpa*$^{-/-}$ genotype. Based on our genotyping, the number of generated male *Ttpa*$^{-/-}$ offspring was similar to the prediction for the E-DOSE diet strategy (27%) but slightly lower for the LOW-E diet strategy (19%). Most female offspring were not genotyped because our vitamin E studies have focused on males thus far. Finno et al. (22) observed significantly reduced male and female *Ttpa*$^{-/-}$ offspring production rates (18%), presumably due to fetal mortality.

Discussion

The goals of this report were to *1*) describe and evaluate the effects of 2 *Ttpa*$^{+/-}$ breeder diet strategies (E-DOSE and LOW-E) on fertility outcomes and *2*) provide recommendations for using each breeding diet approach. Minimizing offspring brain α-T concentrations at weaning is ideal if these animals are to be used for neurological studies. Previously published research and our preliminary results with the CON strategy suggested that low α-T concentrations can be achieved by feeding dams α-T–restrictive diets. This rationale led to the E-DOSE and LOW-E breeder diet schemes. The pregnancy, gestation, and viability indexes were similar between E-DOSE and LOW-E. However, the E-DOSE strategy more effectively reduced α-T concentrations in *Ttpa*$^{-/-}$ and *Ttpa*$^{+/+}$ weanling brains. LOW-E weanling brain α-T concentrations were modestly higher, but this breeder diet strategy led to a dramatically higher offspring yield than for E-DOSE.

Studies looking at nervous system development or short-term feeding regimens would especially benefit from generating *Ttpa*$^{-/-}$ and *Ttpa*$^{+/+}$ weanlings with low tissue α-T concentrations (**Table 2**). Implementing a postweaning α-T depletion phase to reduce brain α-T concentrations would not be feasible in these studies; the depletion phase would necessarily extend past the life stage of interest. AVED symptoms commonly manifest during childhood (6), but there are relatively few animal studies on this topic, perhaps due to study design obstacles. Studying younger animals may also give insight into α-T's mechanism of action and vitamin E intake requirements during childhood and adolescence. Breeder diet strategies such as E-DOSE should facilitate future research in these areas (Table 2).

Low baseline α-T status is also essential when comparing the in vivo effects of natural versus synthetic α-T. Only 1 stereoisomer of α-T exists in nature (*2R, 4′R, 8′R; RRR*), but synthetic α-T is an equimolar mixture of all 8 possible stereoisomers (all-*rac*; *RRR, RRS, RSR, RRS, SSS, SSR, SRS, SRR*) and is less potent than natural α-T (5). Synthetic α-T is commonly used in rodent diets, and lactating animals transfer synthetic α-T stereoisomers to their offspring. This has previously been demonstrated in lactating humans (33, 34). Importantly, tissue stereoisomer profiles in mouse weanlings could influence the results of later studies that use these animals. The considerations of animal age, study duration, and α-T source (Table 2) prompted us to use the E-DOSE diet strategy for our adolescent mouse study (25).

In previous studies, wild-type dams were administered α-T doses at the beginning of gestation to improve reproductive outcomes. For example, administering 0.035-mg/d doses of synthetic α-T for the first 10 d of gestation was sufficient to maintain dams' first pregnancy (18). In another study, a single 0.5- to 1-mg dose of synthetic α-T at the beginning of gestation was adequate for 3- to 6-mo-old dams, but larger doses were needed for 7- to 12-mo-old dams (21). A single dose was rarely enough for producing a second litter, and never a third litter. Dams administered lower α-T doses (<1 mg) tended to have litters with fewer pups (21). For comparison, our E-DOSE strategy provided dams with overall higher amounts of α-T (~0.23 mg synthetic α-T/d assuming 3 g food intake/d).

The E-DOSE approach is extremely resource intensive and may only be feasible or necessary for a limited set of studies, such as those focused on brain development or short-term feeding interventions in young animals. In contrast, the LOW-E diet strategy greatly simplifies the breeding process and hastens the generation of study animals (Table 2). The LOW-E strategy yields weanlings with only modest reductions in brain α-T compared with a CON diet. However, given the continued tissue expansion during growth and the turnover of α-T, LOW-E–generated animals can be used to study α-T deficiency in various tissues over the long term.

E-DOSE and LOW-E were developed for our nervous system–focused studies, but they could also be applied to other types of *Ttpa*$^{-/-}$ mouse studies, such as those focused on the cardiovascular system (13, 35) and immune/inflammatory responses (36, 37). A strength of our approach was using commercially available diets, instead of dosing dams individually. Notably, this is also the first report comparing the effects of 2 different breeder diet strategies on reproductive outcomes for *Ttpa*$^{+/-}$ breeders.

The E-DOSE and LOW-E diet strategies were primarily used to generate animals for future studies. Evaluating fertility outcomes and each strategy's merits became secondary objectives during the breeding process. Therefore, we did not collect some types of data that are common in the reproduction literature (e.g., presence of vaginal plugs). Additionally, we could not determine the exact amount of α-T consumed by dams during each breeding cycle, and it is unknown whether a dam's α-T stores varied from 1 cycle to the next and consequently affected breeding outcomes. Last, we did not directly evaluate male infertility, but it is possible that this and other unknown factors may confound our results.

We implemented 2 diet strategies for generating *Ttpa*$^{-/-}$ and *Ttpa*$^{+/+}$ pups with low α-T status. Our observations highlight the role of vitamin E in murine reproduction and the influence of the dam's diet on offspring outcomes. Establishing breeding methods for *Ttpa*$^{-/-}$ mice will help guide future research focused on vitamin E metabolism, physiology, and functions.

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