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RESEARCH ARTICLE

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Reproductive toxicity assessment of alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride in CD® rats

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Abstract

Aim: To determine the potential of alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) to induce reproductive toxicity in CD® rats in two independent 2-generation reproduction studies conducted according to Good Laboratory Practices and standardized testing guidelines.

Materials and Methods: Male and female rats (parents and offspring) were allowed continual free access to diets containing concentrations of ADBAC (0, 300, 1,000, or 2,000 ppm) or DDAC (0, 300, 750, or 1,500 ppm), beginning with F_0 generation adults at 10 weeks prior to breeding.

Results: No clinical signs of toxicity were observed in parental rats or their offspring in either study. Dietary exposure of parental rats to ADBAC or DDAC at the highest concentrations produced transient decreases in body weight and/or body weight changes with no or minimal corresponding reduction in food consumption. Offspring (F_1 and F_2) in the highest concentration group in each study also exhibited reduced body weights, often with a corresponding reduction in weight change, beginning on postnatal day (PND) 14 through weaning on PND 28. This reduction in pup body weight corresponded to initiation of self-feeding.

Conclusions: Based on reduced body weights, the no observed adverse effect level (NOAEL) for adult and offspring systemic toxicity was 1,000 ppm for ADBAC and 750 ppm for DDAC (equivalent to approximate daily oral doses of 59 and 45 mg/kg/day, respectively). The reproductive and developmental NOAEL for F_0, F_1, and F_2 generation male and female rats was 2,000 ppm for ADBAC and 1,500 ppm for DDAC (equivalent to approximate daily oral doses of 118 and 91 mg/kg/day, respectively).

Abbreviations: ADBAC, alkyl dimethyl benzyl ammonium chloride; ANOVA, analysis of variance; DDAC, didecyl dimethyl ammonium chloride; EPA, Environmental Protection Agency; ECHA, European Chemical Agency; FIFRA, Federal Insecticide Fungicide and Rodenticide Act; GD, gestation day; LD, lactation day; NOAEL, no-observed-adverse-effect level; OECD, Organization for Economic Co-operation and Development; PND, postnatal day; QACs or Quats, quaternary ammonium compounds.

KEYWORDS

alkyl dimethyl benzyl ammonium chloride, developmental toxicity, didecyl dimethyl ammonium chloride, quaternary ammonium compounds, rat, reproduction toxicity

1 | INTRODUCTION

The quaternary ammonium compounds alkyl dimethyl benzyl ammonium chloride (ADBAC; CAS RN 68424-85-1) and didecyl dimethyl ammonium chloride (DDAC; CAS RN 7173-51-5) are substances that disrupt microbial membrane structure and function at relatively low concentrations. Their structures are shown in Figure 1. ADBAC and DDAC are regulated in the United States by the Environmental Protection Agency (EPA) as antimicrobial pesticides defined by EPA as substances "intended to disinfect, sanitize, reduce, or mitigate growth or development of microbiological organisms or to protect inanimate objects, industrial processes or systems, surfaces, water, or other chemical substances from contamination, fouling or deterioration caused by bacteria, viruses, fungi, protozoa, algae, or slime" (EPA, 2019).

Registration requirements for all EPA-registered antimicrobial pesticides include an extensive evaluation of product chemistry, product performance (antimicrobial efficacy), and data from studies to determine potential hazards to humans, domestic animals, and the environment. In the case of both ADBAC and DDAC, which have been registered for decades, extensive datasets have been developed to address acute, subchronic, chronic, developmental, and reproductive toxicity potential. In a companion manuscript (Hostetler, Fisher, & Burruss, 2021), we describe in detail the results of classic developmental toxicity evaluations that followed EPA and OECD guidelines for study design and conduct. The multigeneration reproduction studies described herein were initiated and completed during the period of 1988–1992 in response to the EPA requirement for data on this endpoint. These studies were conducted at the contract research organization, Bushy Run Research Center (Export, PA), by an experienced team of developmental and reproductive scientists and were conducted in compliance with the United States EPA Pesticide Assessment Guideline 83-4 (EPA, 1984a), the Organization for Economic Cooperation and Development (OECD) Test Guideline 416 (OECD, 1983), and EPA, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards (EPA, 1983, 1984b, 1989) that were effective at the time these studies were performed. All of the studies were subject to internal quality assurance and external audits by EPA.

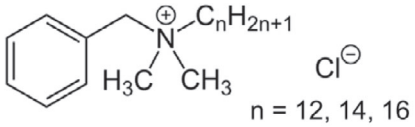
ADBAC and DDAC are considered "active substances" under the principal European regulatory scheme now known as the Biocidal Product Regulation (EU, 2012), and accordingly, these studies are also a key part of the dataset supporting a wide range of biocidal uses in Europe. This work was sponsored by a consortium of quaternary ammonium compound manufacturers[1] and overseen by a third-party toxicology and regulatory consulting company.

2 | MATERIALS AND METHODS

2.1 | Test substances

Alkyl dimethyl benzyl ammonium chloride (ADBAC; CAS RN 68424-85-1) was supplied by Sherex Chemical

Alkyl (C12-C16) dimethyl benzyl ammonium chloride (ADBAC) – CAS RN: 68424-85-1:



Didecyl dimethyl ammonium chloride (DDAC) – CAS RN: 7173-51-5:

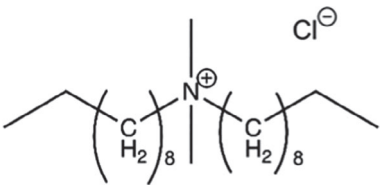


FIGURE 1 Representative structure diagrams for alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC). [Correction added after on 09 October 2021, after first online publication: Figure part for DDAC has been replaced.]

Company (Janesville, WI) as a pale yellow viscous liquid 81.09% concentrate. Likewise, didecyl dimethyl ammonium chloride (DDAC; CAS RN 7173-51-5) was supplied by Lonza Inc. (Fair Lawn, NJ) as a honey-colored, viscous liquid 80.8% concentrate. The primary solvents present in the composition of both test substances were water and ethanol, each at a range of 5–15% of the total composition. The diets were prepared to correct for the percent active ingredient of each test substance.

2.2 | Preparation, administration, and analysis of test diets

ADBAC and DDAC were each incorporated into separate test diets by direct addition of the test substance to the control rat chow (Certified Ground Rat Chow® No. 5002, Ralston-Purina Co., St. Louis, MO) and mixing in a Hobart mixer. This premix was then appropriately diluted with control rat chow and mixed to formulate the high-concentration diets. The mid- and low-concentration diets were prepared by successive dilution and mixing with additional control rat chow (high-concentration diet diluted to formulate mid-concentration diet, mid-concentration diet diluted to formulate low-concentration diet). All test substances and control diets were stored at room temperature. The selected route of administration for this study was dietary because ingestion is a potential route of exposure for humans.

The test diets were analyzed for homogeneity and stability. Both ADBAC and DDAC were found to be uniformly distributed into the test diets using the formulation procedures described above, and were stable for at least 14 days at room temperature in open glass feed containers and for at least 21 days at room temperature in closed polyethylene containers. Based on the stability data, the test and control diets were prepared and distributed to the test or control animals weekly.

The concentration of ADBAC in the test diets was determined using a Water's high-pressure liquid chromatography (HPLC) equipped with a Water's Model 481 Lambda Max Variable Wavelength Detector or Water's 484 Tunable Absorbance Detector and a Water's μBondapak C-18 column (3.9 mm × 30 cm). The concentration of DDAC in the test diets was determined using a Hewlett-Packard 5880A gas chromatography with nitrogen–phosphorus detection. The minimum detection limit was approximately 50 ppm for ADBAC and approximately 5 ppm for DDAC.

Concentration of the test substance in the formulated diets was determined from samples collected weekly from all control and test diets during the first 4 weeks of the study and then every fourth week thereafter. The concentrations of the test diets for both ADBAC and DDAC were analytically confirmed as being within ±10% of target concentrations in all samples taken from the test diets provided to the animals as described. The test substance was not detected in any samples collected from the control diets administered to the control group of either study.

The nominal dietary concentration levels evaluated were 0, 300, 1,000, and 2,000 ppm for ADBAC and 0, 300, 750, and 1,500 ppm for DDAC.

2.3 | Animals, housing, and environmental conditions

Virgin female and male outbred albino CD® (Sprague–Dawley) rats [Crl:CD® (SD)BR] 27 days of age, were obtained from Charles River Breeding Laboratories, Kingston, NY, and were acclimated for approximately 2 weeks. During the 2-week quarantine period, the F_0 parental animals were housed two per cage, by sex, followed by single housing for the duration of the study, except for the cohabitation and lactation periods. The F_1 parental animals were also housed singly following the single housing pattern of the F_0 parental animals. During the quarantine and treatment periods, mating, and most of gestation, rats were housed in stainless steel, wire-mesh cages. From gestation day (GD) 20 through parturition and lactation, female rats were housed in plastic shoebox cages with Alpha-Dri® bedding (Shepherd Specialty Papers, Inc., Kalamazoo, MI).

All animals were housed in environmentally controlled animal rooms set to maintain the temperature at 19–25 °C with relative humidity at 40–70% and a 12-h light/12-h dark cycle. Ralston-Purina Company Certified Ground Rodent Chow® No. 5002 (as control vehicle or mixed with the test substance in the diet) and tap water (Municipal Authority of Westmoreland County, Greensburg, PA) were provided ad libitum throughout the study.

2.4 | Study design

These studies were conducted in compliance with the United States EPA Pesticide Assessment Guidelines (EPA, 1984a), the Organization for Economic Cooperation and Development (OECD) Test Guideline 416 (OECD, 1983), and EPA, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Good Laboratory Practice Standards (EPA, 1983, 1984b, 1989).

Figure 2 provides a graphic representation of the study design for ADBAC and DDAC two-generation

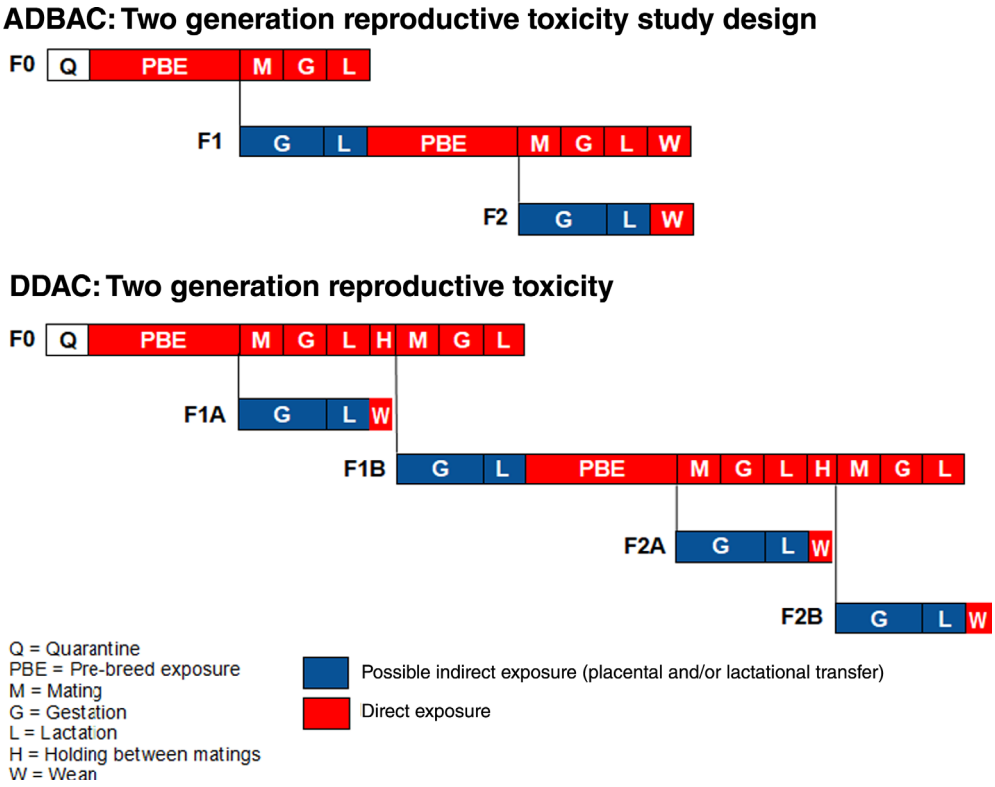


FIGURE 2 Study design of the alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) two-generation reproductive toxicity studies in rats

TABLE 1 Group assignments for two-generation reproduction toxicity studies with alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

Treatment	Group number	Target concentration in diet (ppm)	No. parental males in each generation	No. parental females in each generation
Control diet[a]	1	0	28	28
ADBAC	2	300	28	28
ADBAC	3	1,000	28	28
ADBAC	4	2,000	28	28
Control diet[a]	1	0	28	28
DDAC	2	300	28	28
DDAC	3	750	28	28
DDAC	4	1,500	28	28

[a]Control diet was ground rat chow that was used as the vehicle for the test substance-treated diets.

reproduction studies and group allocations for both studies are presented in Table 1. The first day of the prebreed period for both F_0 and F_1 parental generations is considered Day 0 or the first day of Week 0.

2.4.1 | ADBAC

Target concentrations of ADBAC in the diet were selected based on the results of 14- and 90-day feeding studies with ADBAC in this same strain of rat. Briefly, in an EPA guideline-compliant 90-day dietary toxicity study (OPP Guideline 82-1), rats were exposed to ADBAC in the diet at concentrations of 0, 100, 500, 1,000, 4,000, and 8,000 ppm. Significant mortality (80%–100%) was observed at ≥4,000 ppm and the systemic NOAEL was identified as 500 ppm for males (based on decreased body weight and weight gain at 1,000 ppm) and 1,000 ppm for females (based on deaths at 4,000 ppm and decreased body weight and weight gain for surviving females in this group). Histopathologic evaluations were conducted on all animals that died on the study and on animals in the

control and 1,000 ppm groups. The only treatment-related effects identified were findings related to irritation of the gastrointestinal tract in animals that died in the 4,000 and 8,000 ppm groups. No other pathologic or histopathologic findings in organs distant from the gastrointestinal tract were noted. A subsequent 14-day dietary study was conducted to determine a maximum tolerated dietary concentration between 1,000 and 4,000 ppm to select the highest dietary concentration for the reproductive toxicity study. No mortality occurred in either sex. Clinical signs of toxicity (loose feces), decreased food consumption and body weights, and gross pathology findings (principally distended fluid- and gas-filled viscera), were observed for animals in the 3,000 ppm dose group. An initial trend toward decreased food consumption and body weight gain was observed for males in the 2,000 ppm dose group. This effect was assumed to be related to diet aversion. Females at the 2,000 ppm dose evidenced decreased food consumption only. Treatment-related findings for the animals from the 2,000 ppm group of both sexes at necropsy were limited to dilatation and distension of the cecum in approximately 50% of the animals. After consideration of the above information, the ADBAC test diet concentrations selected for this two-generation reproduction toxicity study were 0, 300, 1,000, and 2,000 ppm.

In the definite study with ADBAC, following the completion of a 2-week acclimation period, male and female rats were allocated to four study groups (28/sex/group) by means of a computer-generated randomization program based on body weight stratification. Selected F_0 animals (28/sex/group) were approximately 6 weeks of age at the initiation of test substance exposure (Day 0). After 10 weeks of exposure to the appropriate test diets, the F_0 animals were paired (1 male to 1 female within each test group) for up to 21 days to produce F_1 litters. F_1 litters were weaned on PND 21 and when the last litter reached PND 28, F_1 offspring (28/sex/group) were selected to constitute the F_1 parental generation. After at least 10 weeks of continued exposure to the test diets (prebreed period), F_1 parental animals were paired (as described above) to produce the F_2 generation. All F_0 and F_1 parental males were necropsied after the completion of parturition and all F_0 and F_1 parental females were necropsied following weaning of the F_1 and F_2 litters, respectively. In addition, selected F_1 pups (10/sex/group) and F_2 pups (10/sex/group) were euthanized on PND 28 and examined macroscopically for gross lesions. Each selection of F_1 and F_2 pups described above was based on a computer-generated random selection scheme. All remaining, unselected F_1 and F_2 pups were examined for gross external abnormalities, euthanized, and discarded.

The test and control diets were initially provided to the F_0 parental animals on the first day of prebreed exposure (Day 0) and remained continuously with the respective groups of animals throughout subsequent generations until study completion. F_0 males and females were exposed to the test diets for 117–118 and 114–133 consecutive days, respectively (i.e., initiation of prebreed through the day prior to terminal necropsy). F_1 parental males and females were exposed to the test diets for approximately 145–152 and 141–167 consecutive days, respectively, (i.e., from weaning on PND 21 through the day prior to terminal necropsy) not including possible exposure during self-feeding which typically begins around PND 14 (Redman & Sweney, 1976).

2.4.2 | DDAC

Target concentrations of DDAC in the diet were selected from previously conducted 14- and 90-day feeding studies in CD (Sprague–Dawley) rats. Briefly, in an EPA guideline-compliant 90-day dietary toxicity study (OPP Guideline 82-1), rats were exposed to DDAC in the diet at concentrations of 0, 100, 300, 600, 1,000, and 3,000 ppm. Mortality (80%) was observed at 3,000 ppm; therefore, the systemic NOAEL was identified as 1,000 ppm. Histopathologic evaluations were conducted on all animals that died on the study and on animals in the control and 1,000 ppm groups. As with ADBAC, histopathologic findings were limited to findings associated with gastrointestinal tract irritation in animals that died on the study at 3,000 ppm. A subsequent 14-day dietary study was conducted to determine a maximum tolerated dietary concentration between 1,000 and 3,000 ppm to select the highest dietary concentration for the reproductive toxicity study. No mortality occurred in either sex. As with ADBAC, clinical signs of toxicity (loose feces), decreased food consumption and body weights, and gross necropsy findings (principally distended fluid- and gas-filled viscera, and abnormal cecal contents), were observed for animals in the 2,000 ppm dose group. An initial trend toward decreased food consumption and reduced body weight gain was observed for males in the 1,500 ppm dose group. This effect was assumed to be related to diet aversion. Females at the 1,500 ppm dose evidenced decreased food consumption and body weights throughout the study. Treatment-related findings for the animals in the 1,500 ppm group of both sexes at necropsy were limited to dilatation and distension of the cecum, and abnormal cecal contents in approximately 33% of the animals. Based on the results of these studies the DDAC test diet concentrations selected for this two-generation

reproduction toxicity study were 0, 300, 750, and 1,500 ppm.

In the definitive study with DDAC, following the completion of a 2-week acclimation period, male, and female rats were allocated to four study groups (28/sex/group) by means of a computer-generated randomization program based on body weight stratification. Selected F_0 animals (28/sex/group) were approximately 6 weeks of age at the initiation of test substance exposure (Day 0). After 10 weeks of exposure to the appropriate test diets, the F_0 animals were paired (one male to one female within each test group) for up to 21 days to produce F_{1A} litters. F_{1A} litters were weaned on PND 21 and selected offspring (10/sex/group) were necropsied at 28 days postpartum. The remaining F_{1A} offspring were examined for gross external anomalies, euthanized, and discarded. A second breeding of the F_0 parental animals commenced at least 14 days after the last weaning of the F_{1A} litters. F_{1B} litters were weaned on PND 21 and on PND 28, F_{1B} offspring were selected for necropsy (10/sex/group) or were selected (28/sex/group) as the parental generation to produce the F_2 generations. All remaining F_{1B} pups were examined for gross external abnormalities, euthanized, and discarded. Animals selected as the F_{1B} parental generation continued to receive the test diets for at least 10 weeks and were then bred, in the same manner as the F_0 generation, to produce F_{2A} and F_{2B} litters. Offspring from these litters were weaned on PND 21 and selected offspring (10/sex/group/generation) were necropsied on PND 28. Each selection of F_1 and F_2 pups described above was based on a computer-generated random selection scheme. All unselected pups were examined for gross external abnormalities, euthanized, and discarded.

Necropsies were performed on all F_0 males and parental F_{1B} males after completion of the second generation delivery period. All F_0 females and parental F_{1B} females were necropsied following weaning of their respective second litters (F_{1B} or F_{2B} litters) on PND 21.

The test and control diets were initially provided to the F_0 parental animals on the first day of prebreed exposure (Day 0) and remained with the respective groups of animals throughout subsequent generations until study completion. The F_0 and F_1 parental males had continual access to the appropriate test diets throughout mating and through the day prior to euthanasia. The F_0 and F_1 parental females continued to receive the appropriate test diets throughout mating, gestation, and lactation, and through the day prior to euthanasia. F_0 males and females were exposed to the test diets for approximately 191–210 consecutive days and F_{1B} parental males and females were exposed to the test diets for approximately 208–244 consecutive days (not including the possible exposure during self-feeding which typically begins around PND 14 (Redman & Sweney, 1976).

2.5 | Parameters evaluated

2.5.1 | F_0 and F_1 parental generations: ADBAC and DDAC

All animals were observed twice daily for mortality and moribundity. Detailed clinical examinations were conducted once daily for all parental animals. These daily clinical examinations included, but were not limited to, changes in skin and fur, eyes, and mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior patterns. Body weights were recorded weekly for all parental male study animals throughout the study duration and weekly for parental females until evidence of successful mating. Once evidence of mating was observed, body weights of F_0 and F_1 parental females were recorded on GD 0, 6, 15, and 20 and on lactation days (LD) 0 (when possible), 7, 14, and 21.

F_0 and F_1 parental male and female food consumption was measured weekly, except during the mating period. Following evidence of successful mating, food consumption of F_0 and F_1 parental females was recorded every 3–4 days through gestation and lactation. After the completion of the mating period, weekly food consumption measurements continued for parental males until termination.

Male and female reproductive performance was evaluated by calculating the male and female mating and fertility indices. All females were allowed to deliver naturally and rear their young to weaning (PND 21). Litters remained together for approximately seven additional days following weaning (PND 28), at which time selection for the next generation and/or terminal necropsy was conducted.

2.5.2 | F_1 and F_2 litters: ADBAC and DDAC

On the day of parturition (PND 0), the numbers of stillborn and live fetuses were recorded and all live pups were examined externally for the presence of malformations and to determine sex. Each litter was examined twice daily throughout the postnatal period for survival, and all deaths were recorded. A necropsy was performed on any pup found dead during the postnatal period to determine (if possible) the cause of death and to identify any abnormalities. Litters were examined daily for any adverse changes in appearance or behavior. On

PND 4, litters were reduced to eight pups (four pups/sex, when possible), using a computer-generated random scheme. Culled pups were examined externally, euthanized, and discarded. On PNDs 1, 4, 7, 14, 21, and 28, clinical observations were performed on each pup, sex was confirmed, and body weights were recorded. The following litter parameters were calculated for each generation: gestational index, live birth index, and postnatal survival indices for the following postnatal intervals: PND 0–4, 4–7, 7–14, 14–21, 21–28, and lactation index (i.e., overall postnatal survival index from PND 4 [post-cull]-PND 21).

2.6 | Necropsy and pathology: ADBAC and DDAC

A complete necropsy was conducted on all F_0 and F_1 parental animals found dead, euthanized in extremis, or at termination. The following tissues were harvested from all parental animals for possible microscopic evaluation: vagina, uterus, ovaries, testes, epididymides, seminal vesicles, prostate, and other tissues with gross lesions. Microscopic examinations were carried out on the selected tissues of all adult rats from the control group and high-concentration test diet group and from adult rats in the low- and mid-concentration test diet groups that died during the study or which had gross lesions. In addition, the testes and epididymides from adult males that failed to sire a litter were also examined microscopically from the low- and mid-concentration test diet groups. All tissues were fixed in 10% neutral-buffered formalin and the uteri from females that did not produce a litter were stained with potassium ferricyanide to confirm pregnancy status and number of implantation sites (if any).

A gross internal examination was also performed on any pup appearing abnormal or dying on test, and on 10 pups per sex from each test group of the F_1 and F_2 generations for the ADBAC study and each test group of the F_{1A}, F_{1B}, F_{2A}, and F_{2B} generations for the DDAC study.

2.7 | Statistical methods: ADBAC and DDAC

Quantitative continuous variables (e.g., body weights, food consumption, and so on) for all test and control groups were subject to Levene's test for equal variances (Levene, 1960), analysis of variance (ANOVA), and *t*-tests. The unit of comparison was the male, the female (during prebreed exposure periods), the pregnant female, or the litter (Weil, 1970). When Levene's test indicated homogeneous variances and the ANOVA was significant, the pooled *t*-test was used for pairwise comparisons. When Levene's test indicated heterogeneous variances, all groups were compared by an ANOVA for unequal variances (Brown & Forsyth, 1974) followed, when necessary, by the separate variance *t* test for pairwise comparisons. The significance levels for the *t*-test comparisons were corrected by the Bonferroni method.

Nonparametric data were statistically evaluated using the Kruskal–Wallis test (Sokal & Rohlf, 1969) followed by the Mann–Whitney *U* test for pairwise comparisons (Sokal & Rohlf, 1969) when appropriate. Frequency data (such as the various indices) were compared using the Fisher's exact test (Sokal & Rohlf, 1969).

3 | RESULTS

The use of the term "significant" or "significantly" indicates a statistically significant change from the corresponding control value at $p < .05$ or $p < .01$. Summary data are tabulated for statistically significant and/or test substance-related differences, unless otherwise noted.

3.1 | ADBAC: F_0 and F_1 parental animals

3.1.1 | Survival and clinical observations

There were no significant test substance-related clinical observations or effects on survival for the ADBAC-exposed F_0 and F_1 parental animals. All F_0 males and females survived to scheduled termination. The number of animals found dead or euthanized in extremis, expressed as a ratio of the total number of animals per group were as follows: 1/28 F_1 males in the 2,000 ppm group (euthanized moribund due to caging accident); 1/28 F_1 females each at 0 and 300 ppm were found dead. Since the two female deaths were in the control and lowest exposure group they were not considered to be related to test substance exposure.

3.1.2 | Body weights and food consumption

There were no significant treatment-related body weight decrements associated with ADBAC ingestion in F_0 and F_1 males throughout the study (Figures S1 and S2). F_0 male body weight change was generally equivalent across all groups with the exception of a single significant reduction observed at 2,000 ppm for Week 4–5 only. A significant decrease in body weight change of the F_1 males in the 2,000 ppm ADBAC group was observed during Week 1–2.

Significant treatment-related reductions in body weights of the F_0 females occurred only in the 2,000 ppm ADBAC group for Weeks 5, 6, 9, and 10 of the pre-breed period (Figure 3) and body weight change for this group was significantly decreased for Week 8–9. Gestational body weights of F_0 females in the 2,000 ppm ADBAC group were significantly reduced only on GD 0 and remained equivalent to the control group values through lactation Day 14 (Figure 4). By lactation Day 21 the body weights were significantly increased in the 2,000 ppm ADBAC group compared to controls (Figure 4). There were no significant decreases in body weight changes throughout the gestation period (Figure S3); however, lactational body weight change was significantly increased throughout lactation (Figure 5).

Body weights and body weight changes of the F_1 females in the 2,000 ppm ADBAC group were not affected throughout the prebreed period, gestation, or lactation (Figure 5; and Figures S3–S5). A significant increase in absolute body weight noted in the F_1 females in the 1,000 ppm ADBAC group compared to controls from prebreed Week 3 through LD 14 was not considered an adverse effect related to ADBAC exposure (Figures S4 and S5).

Food consumption in F_0 males was significantly reduced in the ADBAC 2,000 ppm group for the first week of treatment only that was likely due to test substance palatability. There were irregular reductions in food consumption throughout the study in F_0 and F_1 parental males and females receiving ADBAC at the 2,000 ppm dose level. It was unclear if this represented diet rejection or poor palatability. These reductions were not consistently observed over time nor did they correlate to the timing of reduced body weights mentioned above; therefore, these reductions were not considered an adverse or toxic effect. Other periodic instances of significant increases in food consumption were noted in the 300 and 1,000 ppm ADBAC groups during the F0 and F1

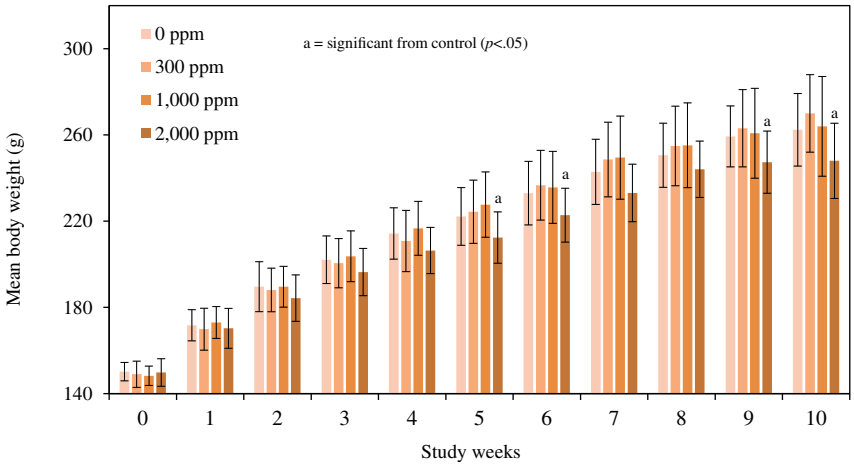


FIGURE 3 F0 parental female prebreed body weights: two-generation reproductive toxicity study of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered via dosed feed to rats (mean ± *SD*)

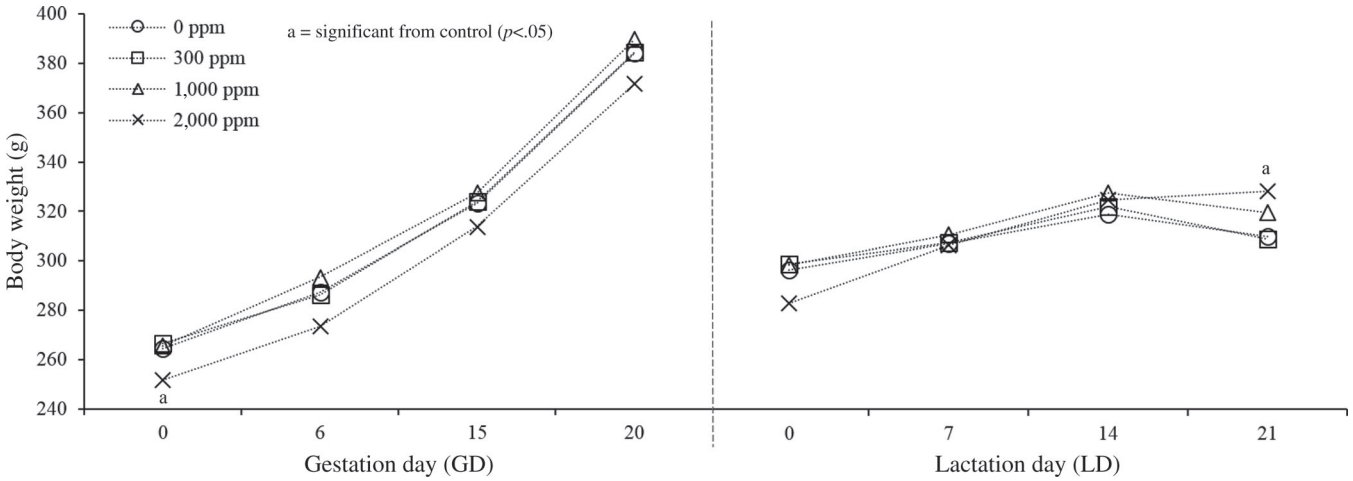


FIGURE 4 F0 female gestation and lactation body weights: two-generation reproductive toxicity study of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered via dosed feed to rats

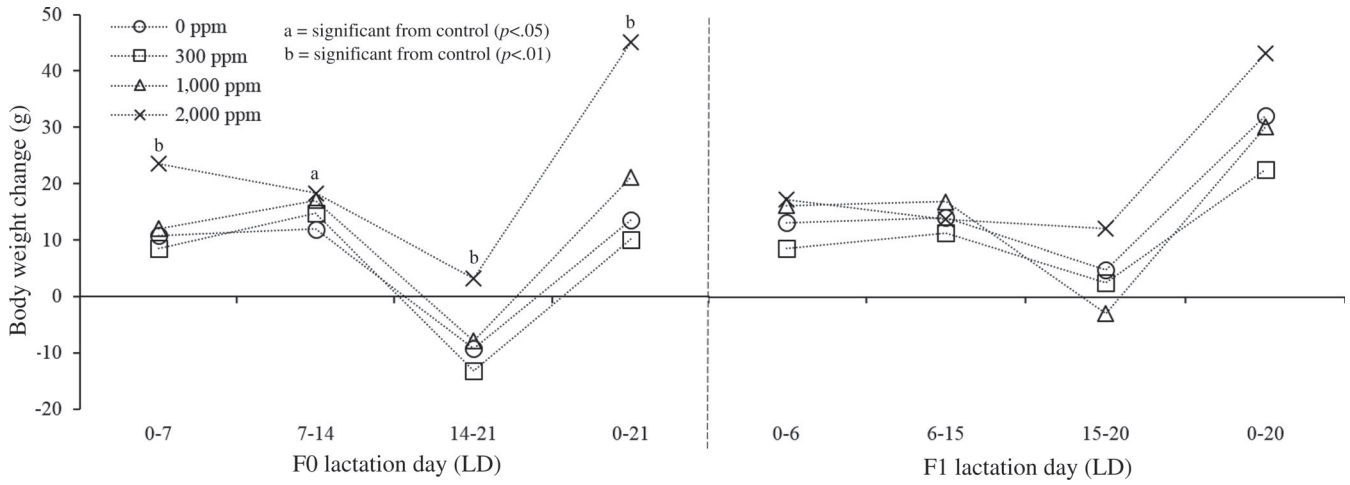


FIGURE 5 F0 and F1 female lactation body weight changes: two-generation reproductive toxicity study of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered via dosed feed to rats

generations prebreed, gestation, or lactation periods and were not considered to be test substance-related.

3.1.3 | Test substance consumption

The calculated doses of ADBAC in mg/kg/day, based on the feed consumed, body weight, and levels of dietary ADBAC (corrected for purity), are presented in Table 2 for F_0 and F_1 males and females during the respective prebreed periods. As expected, because the concentrations of the test substance were held constant throughout the study and because of decreasing food consumed as a function of increasing body weight with age, the calculated doses of ADBAC consumed dropped steadily from Week 1 to 10 in all treated groups. The overall mean amount of ADBAC consumed for the F_0 and F_1 males during the respective prebreed periods were as follows: 16.8 and 15.5 mg/kg/day for the 300 ppm group, 55.3 and 50.7 mg/kg/day for the 1,000 ppm group, and 109.2 and 101.7 mg/kg/day for the 2,000 ppm group. The overall mean test substance consumed for the F_0 and F_1 females during the respective prebreed periods were: 20.7 and 20.1 mg/kg/day for the 300 ppm group, 65.9 and 63.7 mg/kg/day for the 1,000 ppm group, and 133.6 and 127.4 mg/kg/day for the 2,000 ppm group.

3.1.4 | Reproductive performance, gestation length, and parturition

No test substance-related effects were observed on F_0 and F_1 male and female reproductive performance including mating and fertility indices or female gestational index in any ADBAC-treated group. No test substance-related effects were noted on mean gestation lengths or the process of parturition at any dosage/exposure level in the F_0 and F_1 generations (Table 3; and Tables S1 and S2).

3.1.5 | Necropsy and pathology

There were no test substance-related macroscopic or microscopic findings noted for F_0 and F_1 parental males and females at any dosage level after continued access to ADBAC-treated diets. There were no test substance-related macroscopic findings noted at any dosage level in the F_1 and F_2 pups that were found dead or the selected pups (10 pups/sex/group) that were euthanized at the scheduled necropsy on PND 21.

3.2 | ADBAC: F_1 and F_2 litter/pup data

There were no test substance-related effects noted on F_1 and F_2 mean numbers of pups born, live litter size on PND 0, percentage of males, or postnatal survival in any ADBAC-treated group (Tables S3 and S4). Significant reductions in F_1 and F_2 pup body weights were observed at 2,000 ppm on PND 21 and/or PND 28 (1-week post weaning) (Figures 6 and 7). F_1 and F_2 pup body weight changes at 2,000 ppm were significantly reduced for the corresponding time intervals, PND 14–21 and PND 21–28. These late postnatal reductions in body weight observed in the F_1 and F_2 pups are likely a result of reduced diet palatability at 2,000 ppm as the F_1 animals randomly selected as the breeders for the next generation did not show continued significant body weight reductions throughout the prebreed, gestation or lactation periods (see Section 3.1.2). The reductions in F_1 and F_2

TABLE 2 Test substance consumption during the prebreed period (Weeks 1–10) for two-generation reproduction toxicity studies with alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

		Calculated doses received[a] (mg/kg/day)				
Treatment	Target concentration in diet (PPM)	F_0 males	F_0 females	F_1 parental males	F_1 parental females	Males and females combined
ADBAC	300	16.8[b] (13.1–25.1)	20.7 (17.0–26.3)	15.5 (12.3–21.2)	20.1 (17.7–24.1)	18.2[c]
	1,000	55.3 (42.7–83.0)	65.9 (54.3–85.9)	50.7 (40.3–70.9)	63.7 (55.5–75.6)	58.9
	2,000	109.2 (84.9–152.1)	133.6 (112.5–161.0)	101.7 (81.1–139.8)	127.4 (111.3–149.0)	118.0
DDAC[d]	300	17.0 (13.2–25.9)	20.4 (16.0–27.1)	15.7 (12.7–21.5)	18.7 (15.9–24.3)	18.0
	750	42.5 (31.7–63.6)	50.6 (40.3–66.3)	38.9 (30.6–53.2)	47.4 (39.5–59.4)	44.9
	1,500	84.6 (65.4–120.2)	100.9 (82.8–124.4)	81.6 (63.6–113.7)	96.8 (80.3–124.4)	91.0

[a]Calculated dose ranges are approximately based on target ppm concentrations (corrected for test substance purity; 81.09% ADBAC and 80.8% DDAC), and age-dependent body weights and food consumption of the parental animals during the respective 10 week prebreed periods.
[b]Data presented as grand mean and range of means for Weeks 0–10 (prebreed) for each generation and target concentration group.
[c]Values presented as the mean of the grand means for both males and females across both generations and identified as "average representative daily dose."
[d]Values presented for DDAC represent the calculated values for the prebreed period for parental animals from only the first breed and identified as "average representative daily dose."

TABLE 3 Results of reproductive performance of F_0 and F_1 rats at F_1 and F_2 breeds after administration of alkyl dimethyl benzyl ammonium chloride (ADBAC) in the diet

	ADBAC (ppm, in diet)			
Parameter evaluated	0 (control)	300	1,000	2,000
F_0 rats at F_1 breed				
Gestational length (days)[a]	22.0 ± 0.0	22.0 ± 0.0	22.0 ± 0.3	22.0 ± 0.0
Reproductive indices (%)[b]				
Mating index (males and females)	100	100	100	96.4
Fertility index (males and females)	100	96.4	100	100
Gestational index	100	100	100	100
F_1 rats at F_2 breed				
Gestational length (days)[a]	21.9 ± 0.4	22.0 ± 0.4	22.1 ± 0.5	21.9 ± 0.4
Reproductive indices (%)[b]				
Mating index				
(Males)	92.9	85.7	96.4	96.3
(Females)	100	92.6	96.4	92.9
Fertility index				
(Males)	92.3	100	85.2	96.2
(Females)	92.6	96.0	85.2	96.2
Gestational index	100	100	100	100

[a]Values presented as mean gestation days ± *SD*.
[b]Reproductive indices were calculated as follows:

$$\text{mating index males}\,(\%) = \frac{\text{no.of males impregnating females}}{\text{total no.of males paired}} \times 100$$

$$\text{mating index females}\,(\%) = \frac{\text{no.of plug}-/\text{sperm}-\text{positive females}}{\text{total no.of females paired}} \times 100$$

$$\text{fertility index males}\,(\%) = \frac{\text{no.of males siring a litter}}{\text{no.of males impregnating females}} \times 100$$

$$\text{fertility index females}\,(\%) = \frac{\text{no.of pregnant females}}{\text{no.of plug}-/\text{sperm}-\text{positive females}} \times 100$$

$$\text{gestational index}\,(\%) = \frac{\text{no.of females with live litters}}{\text{no.of pregnant females}} \times 100$$

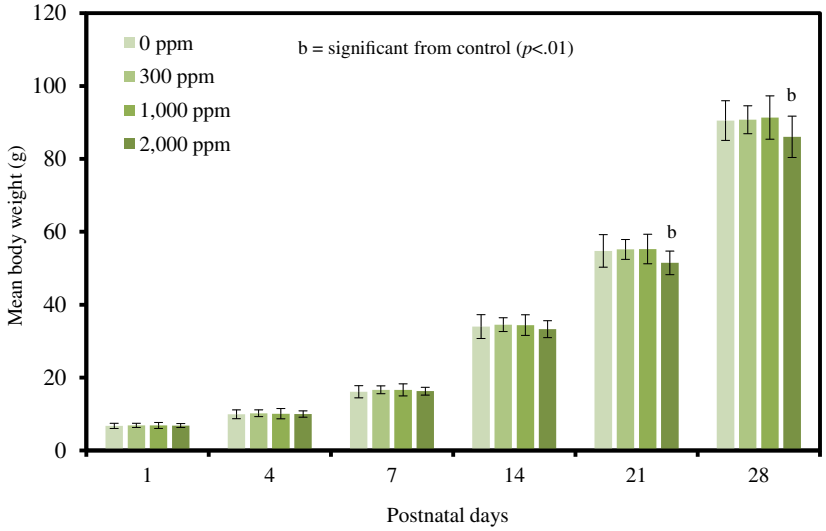


FIGURE 6 F1 pup/litter body weights: two-generation reproductive toxicity study of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered via dosed feed to rats (mean ± *SD*)

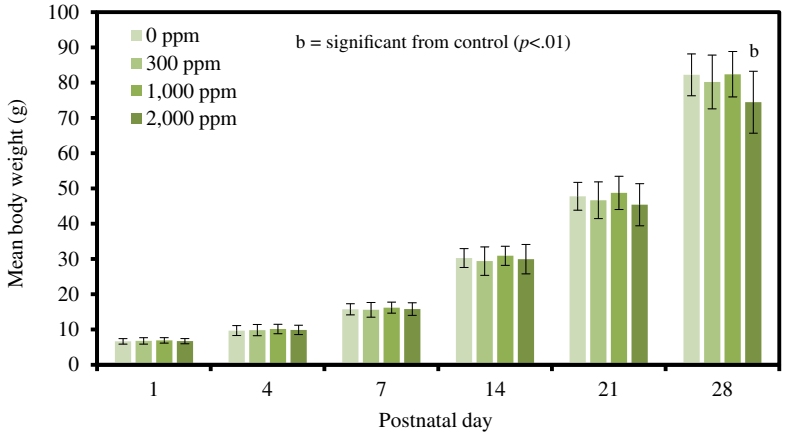


FIGURE 7 F2 pup/litter body weights: two-generation reproductive toxicity study of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered via dosed feed to rats (mean ± *SD*)

pup body weight and/or body weight changes corresponded to initiation of self-feeding, which typically begins around PND 14 (Redman & Sweney, 1976). There were no effects on pup body weights or body weight changes at either generation of pups in the 300 or 1,000 ppm ADBAC groups.

3.3 | DDAC: F_0 and F_1 parental animals

3.3.1 | Survival and clinical observations

There were no significant test substance-related clinical observations or effects on survival for the DDAC-treated F_0 and F_1 parental animals. One F_0 male in the 750 ppm group was euthanized in a moribund condition. At necropsy, multiple calculi were noted in the urinary bladder which may have been attributed to the moribund condition. This finding was not considered to be test substance-related. One F_0 male each in the 300 and 1,500 ppm groups was euthanized after inadvertent trauma unrelated to test substance exposure. All F_0 females and F_1 adult males and females survived to scheduled termination.

3.3.2 | Body weights and food consumption

Systemic toxicity of DDAC to adult rats was evidenced at 1,500 ppm by significant reductions in body weights (Figures 8 and 9), body weight changes and food consumption in the F_0 adult males and females during the non-reproductive phases of the study. Significant reductions were observed in the F_0 female gestational body weights at 1,500 ppm DDAC during the gestation period of the F_{1A} generation (Figure 10); however, the F0 female gestational body weights were not significantly reduced during the gestation period of the F_{1B} generation (Figure 11). In contrast, gestational body weight changes of the F_0 females in the 1,500 ppm DDAC group during the F_{1A} and F_{1B} gestation periods were equivalent to the control group or slightly increased (Figure 12). Similarly,

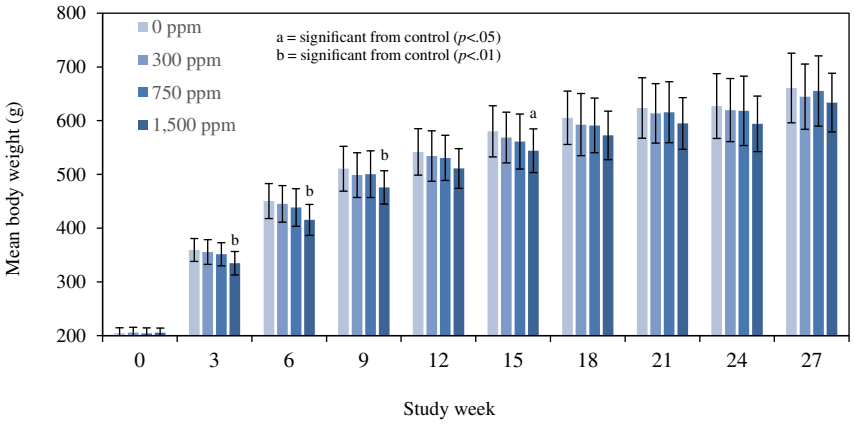


FIGURE 8 F0 parental male body weights: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats (mean ± *SD*)

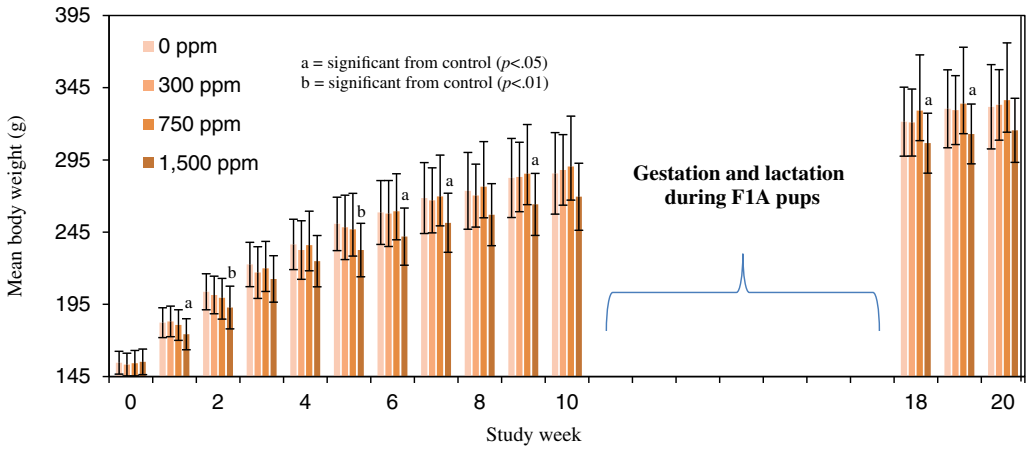


FIGURE 9 F0 parental female prebreed body weights: Two-generation reproductive toxicity study of DDAC administered via dosed feed to rats (mean ± *SD*)

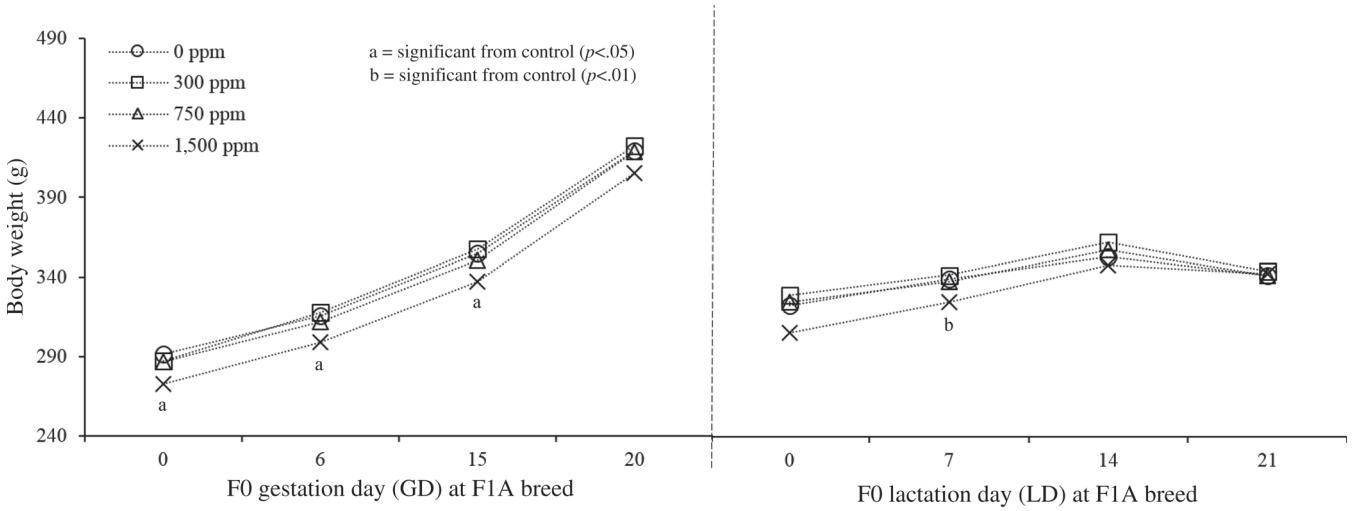


FIGURE 10 F0 parental female gestation and lactation body weights at F1A breeding period: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

lactational body weights of F_0 females were slightly reduced during early lactation of both F_{1A} and F_{1B} litters (Figures 10 and 11), but lactational body weight changes were significantly increased relative to controls (Figure 13). These data indicate that although the amount of weight gained during these phases was

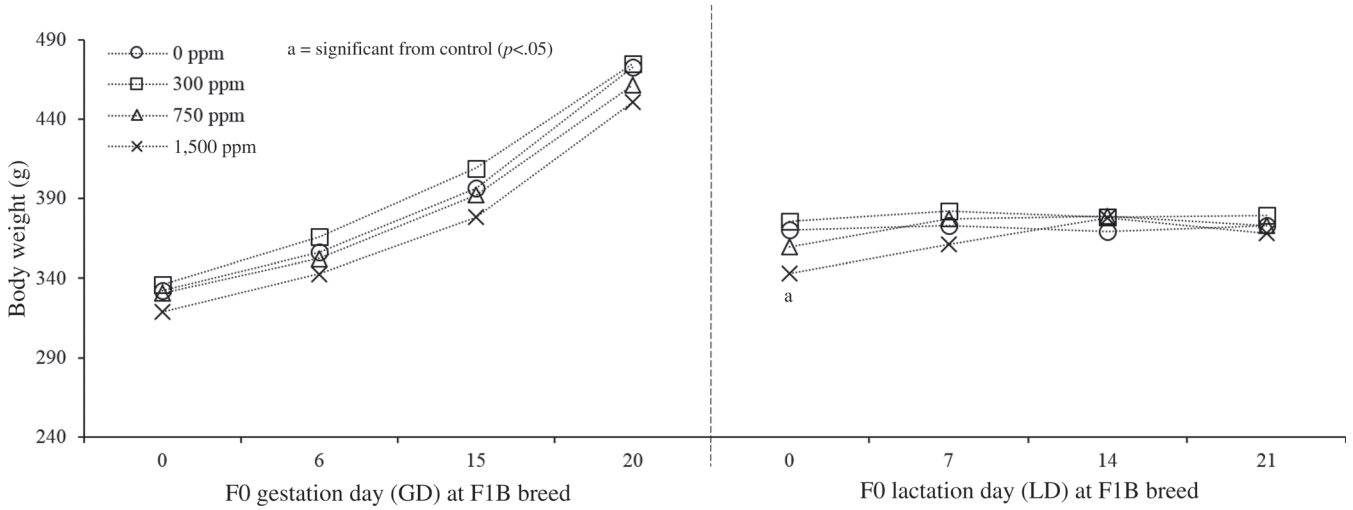


FIGURE 11 F0 parental female gestation and lactation body weights at F1B breeding period: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

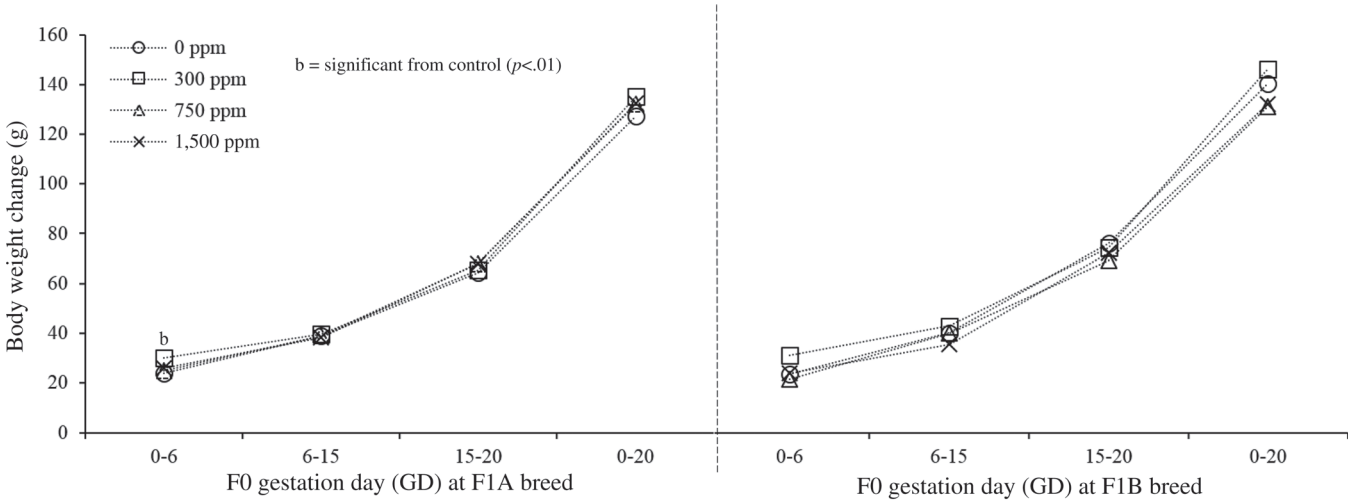


FIGURE 12 F0 parental female gestation body weight changes at F1A and F1B breeding periods: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

equivalent to or greater than that of controls, F_0 females entering gestation and lactation with reduced body weights did not reach control weight values until the latter half of lactation (PND 14 and 21).

Body weights (Figures 14 and 15), body weight changes, and food consumption for the F_{1B} parental males and females in the 1,500 ppm DDAC group were significantly reduced compared to controls throughout the prebreed period. However, at lower concentrations (300 and 750 ppm), body weights were significantly increased in the F_{1B} parental males and slightly increased in the females. The significantly reduced body weights seen in these F_{1B} animals at 1,500 ppm began during the postnatal period, beginning on PND 21 (see Section 3.4 and Figure 19). Body weights and body weight changes of F_{1B} adult females during F_{2A} and F_{2B} gestation and lactation exhibited a similar pattern to that observed in F_0 females during F_{1A} and F_{1B} gestation and lactation. The absolute body weights on gestation day 0 and lactation day 0 were significantly reduced in the F_{1B} adult females in the 1,500 ppm DDAC dose group (Figures 16 and 17). However, the gestation body weight changes of F_{1B} adult females in this dose group were comparable to those of controls (Figure S6), while the lactation body weight changes in this dose group were significantly increased relative to controls (Figure 18). Body weights and body weight changes of F_{1B} adult females during F_{2A} and F_{2B} gestation and lactation were not affected at lower concentrations (300 and 750 ppm).

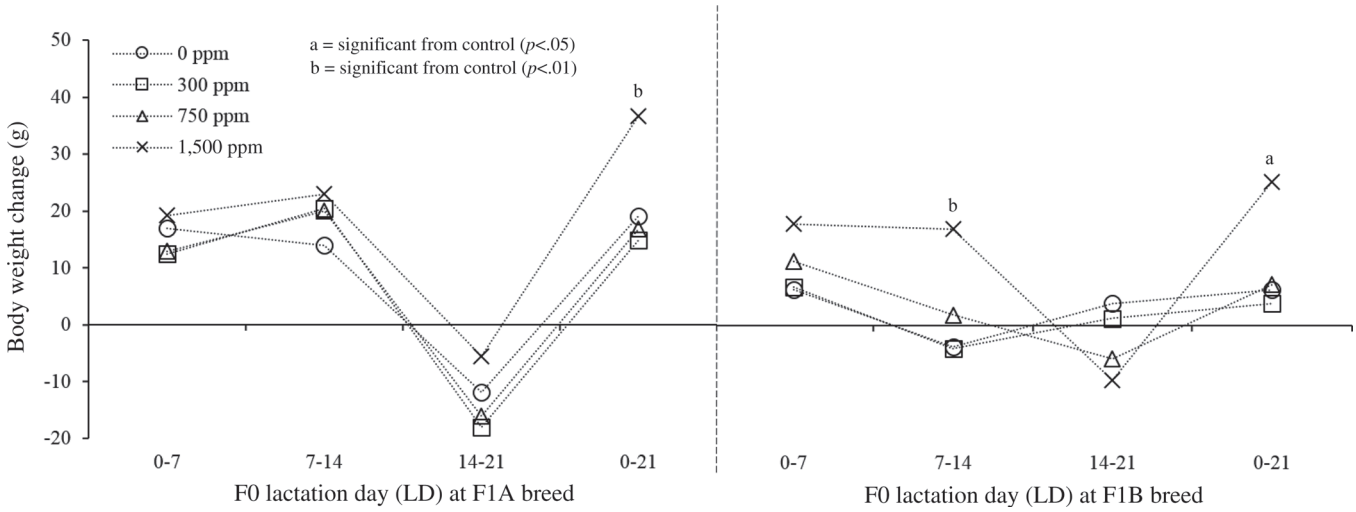


FIGURE 13 F0 parental female lactation body weight changes at F1A and F1B breeding periods: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

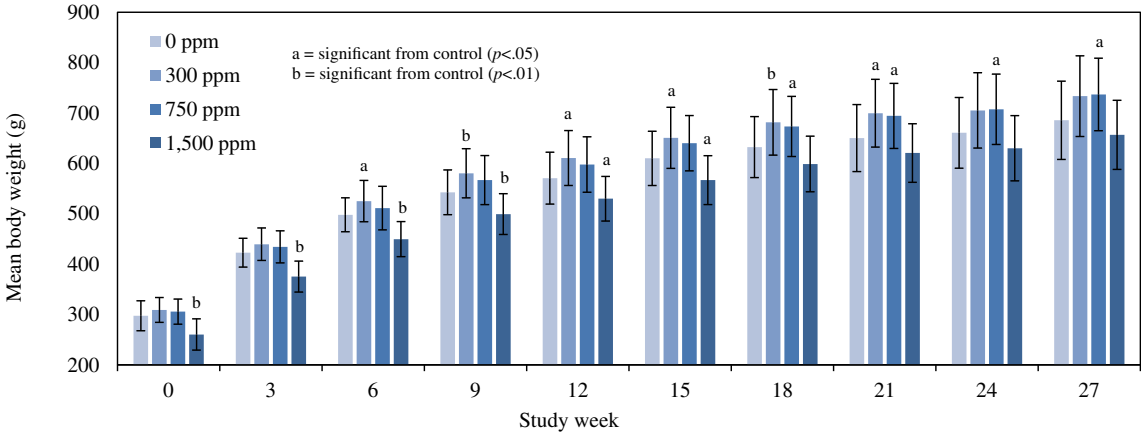


FIGURE 14 F1B parental male body weights: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats (mean ± *SD*)

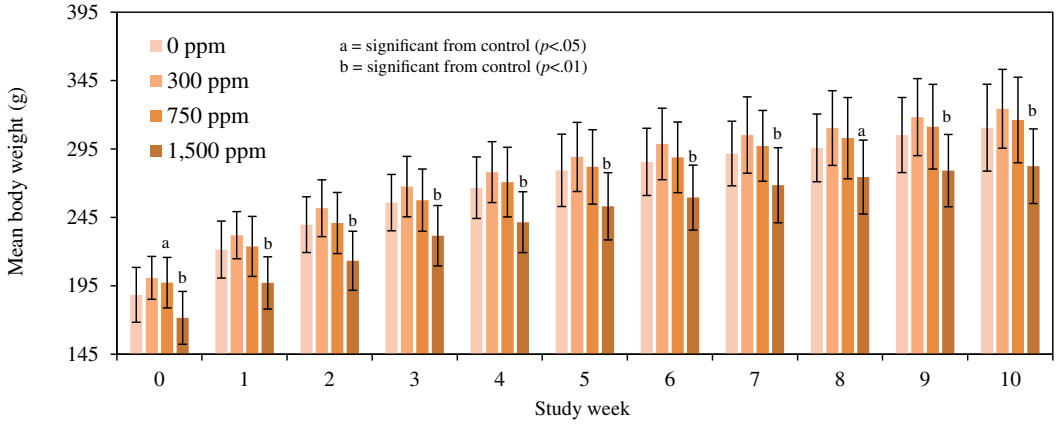


FIGURE 15 F1B parental female prebreed body weights: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats (mean ± *SD*)

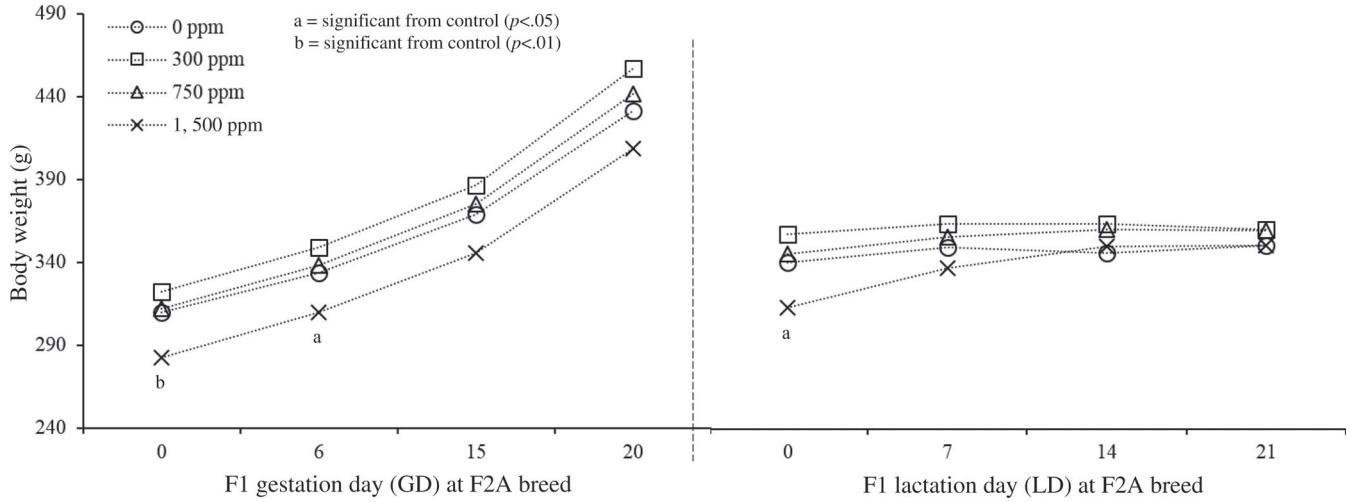


FIGURE 16 F1B parental female gestation and lactation body weights at F2A breeding period: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

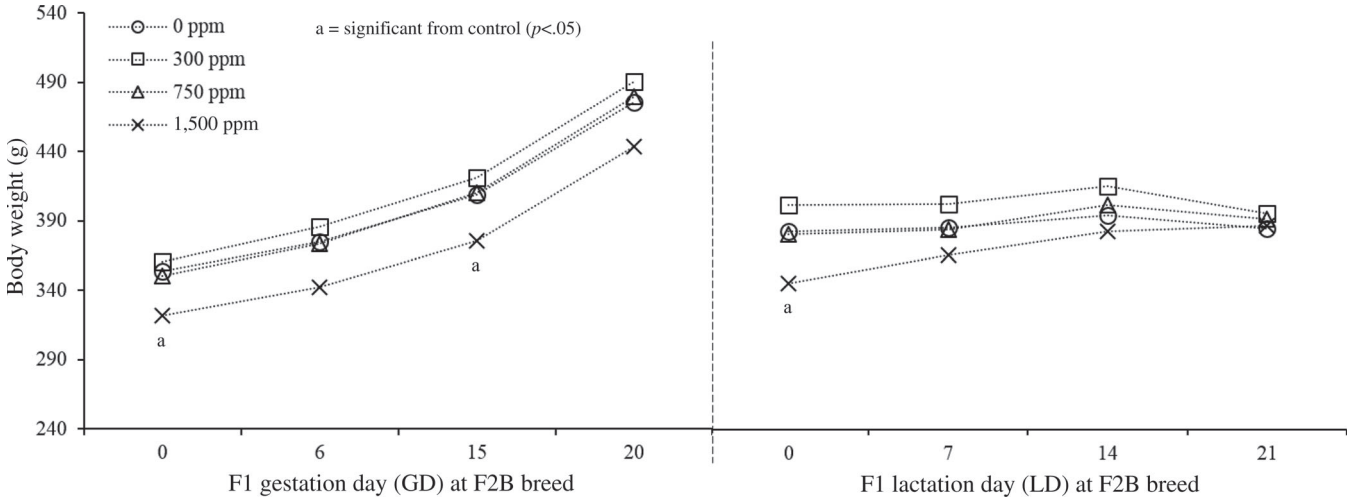


FIGURE 17 F1B parental female gestation and lactation body weights at F2B breeding period: two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

3.3.3 | Test substance consumption

The calculated doses of DDAC in mg/kg/day, based on feed consumed, body weight, and levels of dietary DDAC (corrected for purity), are presented in Table 2 for F_0 and F_1 males and females during each respective prebreed period. As expected, because of decreasing food consumed as a function of increasing body weight with age, the calculated doses of DDAC consumed dropped steadily from Weeks 1 to 10 in all treated groups. The overall mean amount of DDAC consumed for the F_0 and F_1 males during the respective prebreed periods were as follows: 17.0 and 15.7 mg/kg/day for the 300 ppm group, 42.5 and 38.9 mg/kg/day for the 750 ppm group, and 84.6 and 81.6 mg/kg/day for the 1,500 ppm group. The overall mean test substance consumed for the F_0 and F_1 females during the respective prebreed periods were: 20.4 and 18.7 mg/kg/day for the 300 ppm group, 50.6 and 47.4 mg/kg/day for the 750 ppm group, and 100.9 and 96.8 mg/kg/day for the 1,500 ppm group.

3.3.4 | Reproductive performance, gestation length, and parturition

No test substance-related effects were observed on the F_0 and F_1 male and female reproductive performance throughout the study including mating and fertility indices or female gestational index in any DDAC-treated group. No test substance-related effects were noted on

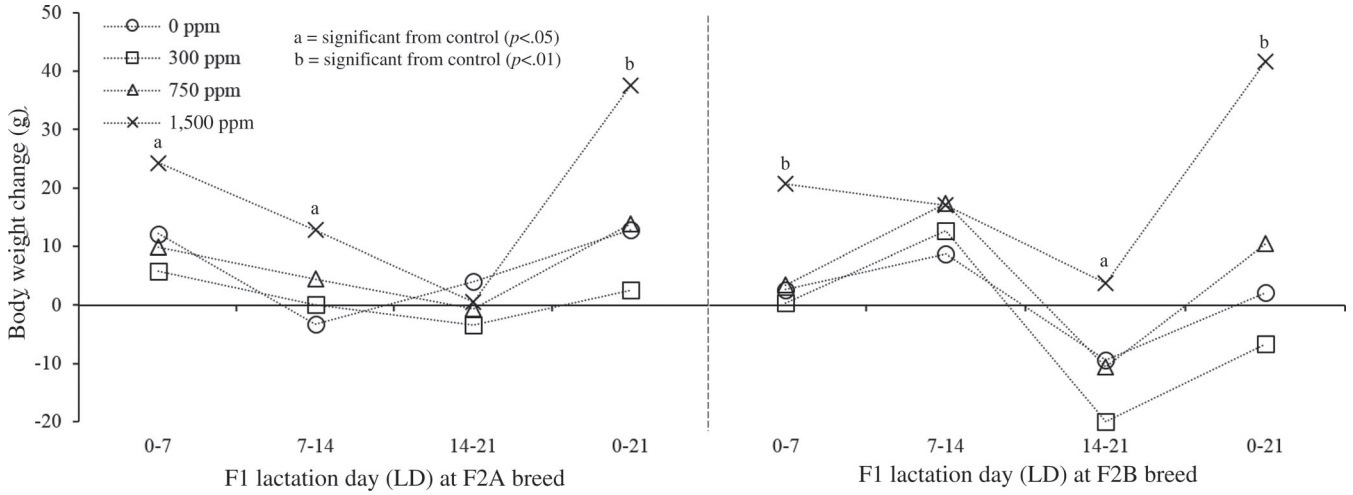


FIGURE 18 F1B parental female lactation body weight changes at F2A and F2B breeding periods: Two-generation reproductive toxicity study of didecyl dimethyl ammonium chloride (DDAC) administered via dosed feed to rats

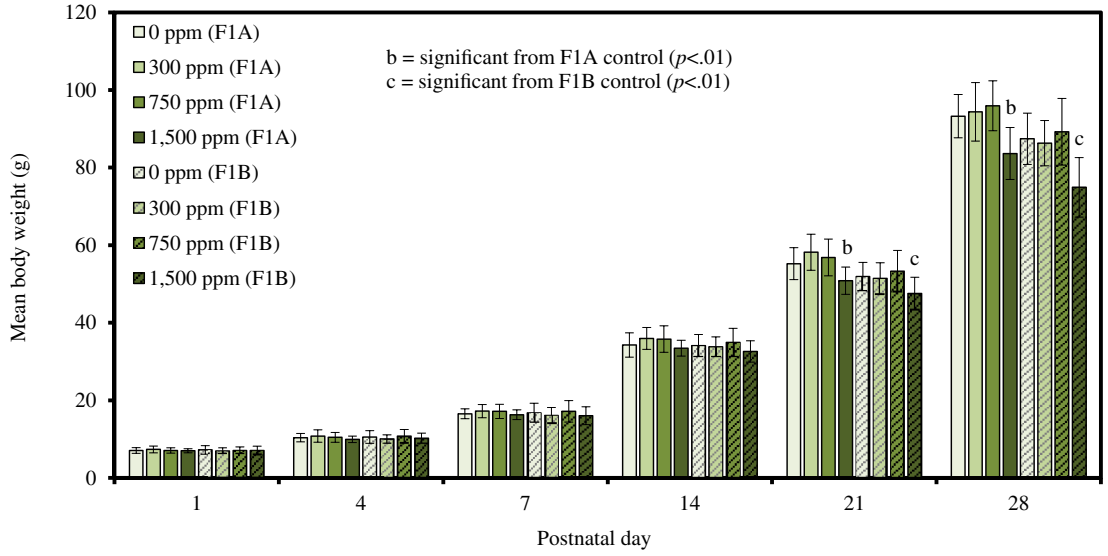


FIGURE 19 F1A and F1B pup/litter body weights: two-generation reproductive toxicity study of DDAC administered via dosed feed to rats (mean ± *SD*)

mean gestation lengths or the process of parturition at any dosage/exposure level in the F_0 and F_1 generations (Tables 4 and 5; and Tables S5–S8).

3.3.5 | Necropsy and pathology

There were no test substance-related macroscopic or microscopic findings noted for F_0 and F_1 parental males and females at any dosage level after continued access to DDAC-treated diets at any concentration.

There were no test substance-related macroscopic findings noted at any dosage level in the F_{1A}, F_{1B}, F_{2A}, or F_{2B} pups that were found dead or the selected pups (10 pups/sex/group/generation) that were euthanized at the scheduled necropsy on PND 21.

3.4 | DDAC: F_{1A}, F_{1B}, F_{2A}, and F_{2B} litter/pup data

There were no test substance-related effects noted on mean numbers of pups born, live litter size on PND 0, percentage of males, or postnatal survival of F_1 or F_2 pups at either breeding interval in any DDAC-treated groups (-Tables S9–S12). Significant reductions in F_{1A} and F_{1B} pup body weights and body weight changes were observed only at 1,500 ppm DDAC on PND 21 and 28 (Figure 19).

TABLE 4 Results of reproductive performance of F_0 rats at the F_{1A} and F_{1B} breed with the administration of DDAC in the diet

	DDAC (ppm, in diet)			
Parameter evaluated	**0 (control)**	**300**	**750**	**1,500**
F_0 rats at F_{1A} breed				
Gestational length (days)[a]	22.2 ± 0.5	22.2 ± 0.4	21.9 ± 0.4	22.1 ± 0.4
Reproductive indices (%)[b]				
Mating index (males and females)	96.4	100	100	100
Fertility index (males and females)	81.5	85.7	71.4	96.3
Gestational index	100	100	100	100
F_0 rats at F_{1B} breed				
Gestational length (days)[a]	22.1 ± 0.4	22.1 ± 0.3	22.0 ± 0.3	22.3 ± 0.4
Reproductive indices (%)[b]				
Mating index				
(males)	92.9	100	100	100
(females)	96.4	96.4	100	96.4
Fertility index				
(males)	73.1	74.1	64.3	85.2
(females)	74.1	74.1	64.3	85.2
Gestational index	100	100	100	100

[a]Values presented as mean ± *SD*.

[b]Reproductive indices were calculated as follows:

$$\text{mating index males}\,(\%) = \frac{\text{no.of males impregnating females}}{\text{total no.of males paired}} \times 100$$

$$\text{mating index females}\,(\%) = \frac{\text{no.of plug}-/\text{sperm}-\text{positive females}}{\text{total no.of females paired}} \times 100$$

$$\text{fertility index males}\,(\%) = \frac{\text{no.of males siring a litter}}{\text{no.of males impregnating females}} \times 100$$

$$\text{fertility index females}\,(\%) = \frac{\text{no.of pregnant females}}{\text{no.of plug}-/\text{sperm}-\text{positive females}} \times 100$$

$$\text{gestational index}\,(\%) = \frac{\text{no.of females with live litters}}{\text{no.of pregnant females}} \times 100$$

F_{2A} pup body weights were equivalent across all groups through PND 21 (day of weaning). On PND 28, F_{2A} pup body weights were significantly reduced at 1,500 ppm (Figure 20) and pup weight changes were reduced from PND 14–28 at 1,500 ppm. The F_{2B} pup body weights were equivalent through PND 14 for all groups. At weaning (PND 21) and on PND 28 the F_{2B} pups body weights were reduced at 1,500 ppm (Figure 20). Pup body weight changes were also reduced in these pups from PND 4–7 and PND 14–28 at 1,500 ppm. There were no reductions in pup body weights or body weight changes at either generation of pups in the 300 or 750 ppm DDAC groups.

4 | DISCUSSION AND CONCLUSION

ADBAC and DDAC were evaluated in EPA and OECD guideline-compliant two-generation reproduction toxicity studies. Test substance concentrations in the diet, which differ for the two substances, were selected based on the results of prior dietary exposure evaluations lasting from 14 to 90 days that identified dietary concentrations that produced adverse effects (reduced body weights or effects on body weight changes) at the higher end of the tested range. Test substance consumption estimates, reported in mg/kg/day, were calculated for each sex and generation, and were corrected for test substance purity. For simplicity and purposes of this discussion, a combined average daily dose for all sexes and generations will be identified as the "average representative daily dose." For ADBAC, the animals in the 300, 1,000, and 2,000 ppm groups received an average representative daily dose of 18, 59, and 118 mg/kg, respectively. For DDAC, the animals in the 300, 750, and 1,500 ppm groups received an average representative daily dose of 18, 45, and 91 mg/kg, respectively.

Under the exposure conditions of these studies, none of the measures of reproductive performance in rats were

TABLE 5 Results of reproductive performance of F_{1B} rats at the F_{2A} and F_{2B} breed with administration of didecyl dimethyl ammonium chloride (DDAC) in the diet

	DDAC (ppm, in diet)			
Parameter evaluated	**0 (control)**	**300**	**750**	**1,500**
F_{1B} rats at F_{2A} breed				
Gestational length (days)[a]	22.1 ± 0.3	22.1 ± 0.5	22.0 ± 0.4	22.1 ± 0.5
Reproductive indices (%)[b]				
Mating index (males and females)	100	96.4	96.4	100
Fertility index (males and females)	71.4	74.1	92.6	92.9
Gestational index	100	100	100	100
F_{1B} rats at F_{2B} breed				
Gestational length (days)[a]	22.3 ± 0.5	22.1 ± 0.4	22.0 ± 0.0	22.2 ± 0.4
Reproductive indices (%)[b]				
Mating index				
(Males)	100	89.3	96.4	100
(Females)	100	85.7	96.4	100
Fertility index				
(Males)	67.9	62.5	77.8	92.9
(Females)	67.9	64.0	77.8	92.9
Gestational index	100	100	100	100

[a]Values presented as mean ± *SD*.

[b]Reproductive indices were calculated as follows:

$$\text{mating index males}\,(\%) = \frac{\text{no.of males impregnating females}}{\text{total no.of males paired}} \times 100$$

$$\text{mating index females}\,(\%) = \frac{\text{no.of plug}-/\text{sperm}-\text{positive females}}{\text{total no.of females paired}} \times 100$$

$$\text{fertility index males}\,(\%) = \frac{\text{no.of males siring a litter}}{\text{no.of males impregnating females}} \times 100$$

$$\text{fertility index females}\,(\%) = \frac{\text{no.of pregnant females}}{\text{no.of plug}-/\text{sperm}-\text{positive females}} \times 100$$

$$\text{gestational index}\,(\%) = \frac{\text{no.of females with live litters}}{\text{no.of pregnant females}} \times 100$$

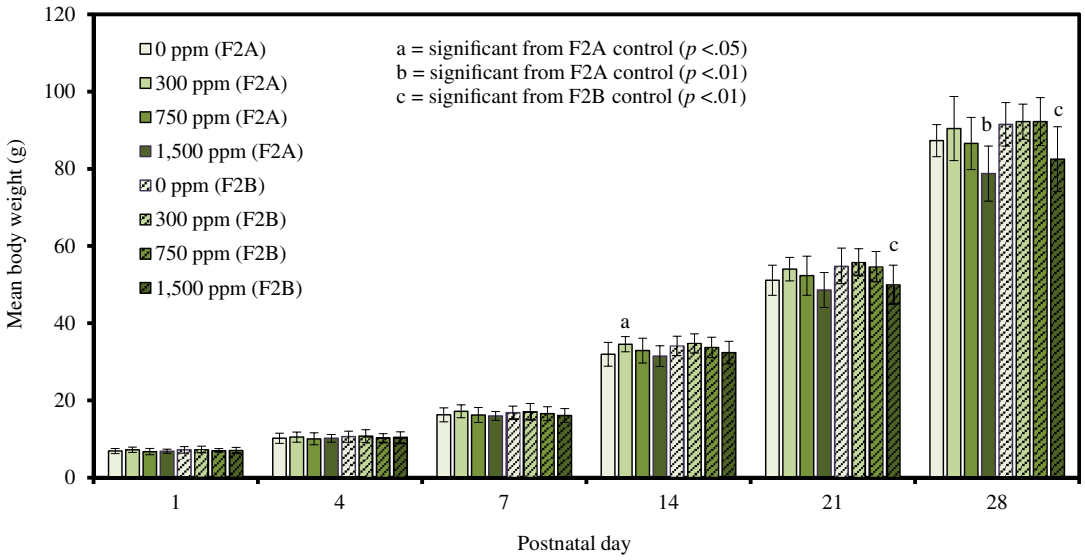


FIGURE 20 F2A and F2B pup/litter body weights: two-generation reproductive toxicity study of DDAC administered via dosed feed to rats (mean ± *SD*)

affected by ADBAC or DDAC. Indices for mating (males and females), fertility (males and females), and gestation, in animals consuming ADBAC or DDAC did not differ from control indices for the F_0, F_1, or F_2 animals. Neither did pup survival indices from birth although PND 28, which ranged from 91.8% to 100%, differ among the ADBAC- or DDAC-treated animals compared with the corresponding controls in the F_1 or F_2 generations. Dietary exposures to ADBAC and DDAC were without effect on the mean number of pups born live, litter size, percentage of males or postnatal survival.

For ADBAC, reductions in F_1 pup body weights were observed at the highest concentration, 2,000 ppm, or approximately 118 mg/kg/day. Likewise, for DDAC, reductions in body weight in the F_0 males and females were observed at the highest concentration (1,500 ppm), approximately 91 mg/kg/day. In developmental (teratology) studies with oral gavage administration of ADBAC and DDAC in rats and rabbits that were conducted just prior to the studies being reported here, the principal adverse findings noted at necropsy were ulceration of the stomach, sloughing of the esophageal lining, and hemorrhage in the stomach, which are hallmark indicators of irritation and/or corrosion (Hostetler et al., 2021). Gastric irritation or corrosion is the probable key adverse outcome leading to reductions in body weight and body weight changes in paternal animals in this study. In addition, rejection of the diet based on reduced palatability may have played a role. Full histopathological examinations were not required by the existing guidelines and were not conducted in these multigeneration reproduction studies. However, it is noteworthy that no macroscopic findings were reported in animals of any generation in either study.

Systemic toxicity was limited to reductions in body weights and body weight gains for both parents and offspring at the highest dietary concentrations of ADBAC and DDAC. Therefore, the NOAEL for adult and offspring systemic toxicity was 1,000 ppm (approximately 59 mg/kg/day) for ADBAC and 750 ppm (approximately 45 mg/kg/day) for DDAC. As there were no reproductive or developmental toxicity effects noted in either study, the NOAEL for reproductive and developmental toxicity was determined to be 2,000 ppm (approximately 118 mg/kg/day) for ADBAC and 1,500 ppm (approximately 91 mg/kg/day) for DDAC.

Based on their intended uses in food contact areas, and thus, the potential for low-level human exposure, EPA has made multiple risk determinations during the course of various regulatory reviews for ADBAC and DDAC. The two studies described in this report are specifically referenced in EPA summaries of their reviews of the available datasets. The most recent publication of EPA's review and conclusions regarding ADBAC and DDAC as potential reproductive toxicants are the respective Final Work Plans (EPA, 2017a, 2017b). In addition to these reviews, EPA has also published their conclusions that resulted from specific regulatory actions and decisions, including the publication of tolerance exemptions for the uses of ADBAC, DDAC, and structurally related substances in food contact areas (EPA, 2017a, 2017b). In all of these cases, the regulatory evaluations not only confirmed the adequacy and completeness of the available database for reproductive toxicity, but also confirmed that the studies described are acceptable for human risk assessment purposes.

In contrast to these definitive studies, there have been reports of non-guideline studies in which pregnancy outcomes and fertility assessments in mice were adversely affected. Melin et al. (2014) administered an ADBAC–DDAC mixture of up to 120 mg/kg to mice in the feed for up to 6 months resulting in maternal toxicity, including decreases in fertility and fecundity, increased time to first litter, longer pregnancy intervals, fewer pups per litter and fewer pregnancies. The relevance of the decreases in fertility and fecundity in mice exposed for up to 6 months at doses known to be toxic in other species remains uncertain but highly questionable. As described in a letter to the editor of the publishing journal (Hostetler, 2014), insufficient data were reported to rule out maternal toxicity as a confounding factor. Numerous other shortcomings were identified. It is noteworthy that these same authors egregiously and erroneously state that "it is possible for chemicals to become widely used in the U.S., despite the fact that their potential reproductive effects have not been directly assessed" and that "quaternary ammonium compound (QAC) disinfectants are one such class of chemicals" (Melin et al., 2014). In a subsequent study, Melin et al. (2015) reported that an ADBAC–DDAC combination treatment in mice for intervals of 5–8 weeks at concentrations of up to 120 mg/kg/day was associated with altered ovulation, oocyte implantation, and estrous cycling. In male mice, changes in sperm concentration, motility, but not viability, were attributed to a different treatment regimen. Absent from these studies was a discussion of dose selection and justification. These investigators also worked with small (relative to guideline studies) sample sizes of mice, which is not the recommended species of choice for reproductive toxicity testing.

The hazard potential of ADBAC, DDAC, and all quaternary ammonium compounds—point of contact irritation—is well documented and is understood to be associated with their chemical structure. In the case of dietary exposures to ADBAC and DDAC in test species (rats, rabbits, dogs, as examples), gastrointestinal

irritation occurs and is both time and concentration-dependent. If irritation proceeds to corrosion, the absorptive profile of the substance could be altered and actually limit systemic exposure. When determining whether a potentially hazardous substance presents an unacceptable risk to human health when used as directed, a fundamental requirement is to also evaluate the exposure potential of the substance in question.

ADBAC and DDAC are formulated into end-use products that are effective in controlling microbial growth at relatively low concentrations. ADBAC and DDAC products intended for food contact surfaces (without a rinse) typically contain not more than 400 ppm (0.04%) active ingredients. They are never intended to be directly ingested. However, in performing well-informed risk assessments, human exposure potential must be considered. In EPA's most recent published exposure estimates (EPA, 2017a, 2017b), average daily exposure to ADBAC and DDAC differ for various human population subgroups. In a relevant and illustrative example, using conservative assumptions built into their exposure models, US EPA estimated that consumer uses of an ADBAC product with a higher than typical (4,900 ppm) concentration result in very low (0.0159 mg/kg) exposures in females aged 13–49 years of age. DDAC exposures resulting from the use of a 240 ppm product in the same population subgroup are approximately three-fold lower (0.00522 mg/kg).

Risk estimates are calculated as margins of exposure (MOE), the ratio of the no-observed-adverse-effect-level obtained from animal toxicology studies to the predicted or estimated human exposure level. For chronic exposures, EPA identified target MOEs of 100 for both ADBAC and DDAC. For the multigeneration reproduction studies described in this report and for the adverse effect associated with reduced body weight (not reproductive performance), the calculated MOEs range from 2,830 to 11,302, far exceeding the EPA targets.

ADBAC was first registered as an active antimicrobial pesticide ingredient in the United States in 1947 and the first DDAC-containing pesticide product was registered in 1962 (EPA, 2017a, 2017b). During this time period, requirements for antimicrobial pesticide active ingredients were established to evaluate their potential to cause adverse effects on health and the environment. Since the early 1980s, regulatory testing guidelines for antimicrobial pesticides have included evaluations for determining the potential of substances to affect reproduction (EPA, 1984a). Accordingly, substantial data sets for ADBAC and DDAC describing environmental fate and effects, ecotoxicity, toxicity (including acute, subchronic, chronic, developmental, and reproductive toxicity), were developed during the late 1980s and throughout the 1990s and beyond. Periodic reviews of the adequacy of the ADBAC and DDAC datasets have been conducted to ensure that registered uses do not present unacceptable risks. Specific regulatory reviews of food contact antimicrobials have occurred to establish tolerances or tolerance exemptions. These exercises consider not only the hazard potential of the active ingredients, but also consider human exposure potential. Thus, the Agency's conclusions are risk-based assessments dependent on the existing toxicology evidence and the known or conservatively estimated potential for human exposure. Food contact uses of ADBAC and DDAC in Europe are authorized by the European Chemicals Agency (ECHA) within the Biocidal Products Regulation. Evaluations and decisions are conducted by the evaluating competent authority, consisting of experts from EU Member States. The dietary risk assessments conducted for European uses typically mirror closely those of the EPA.

EPA and ECHA have consistently concluded that, based on a strikingly similar profile in biological activity, ADBAC and DDAC are acute irritants (corrosive in concentrate form) at the point of contact, independent of species or route of administration. When administered via the diet for extended periods of time (such as in subchronic and chronic studies), ADBAC and DDAC are well tolerated up to a clear threshold dose or concentration, after which they produce a dose- and time-related gastric/gastrointestinal irritation, weight loss, and occasional secondary clinical chemistry changes. Transient or inconsistent food consumption and body weight changes were observed in the ADBAC reproduction study at the 2,000 ppm treatment level, but no clear pattern emerged. Based on findings from numerous studies in multiple species, ADBAC and DDAC do not produce adverse effects in target organs distant from the site of administration and absorption (ECHA, 2015a, 2015b; EPA, 2017a, 2017b).

In conclusion, ADBAC and DDAC have been thoroughly evaluated in guideline-compliant multigenerational studies in rats that revealed no effects on reproduction or fertility. These results are consistent with the behavior of these compounds as direct-acting irritants that do not produce target organ toxicity distant from their point of contact. Confidence in estimates of limited human exposure to ADBAC and DDAC when used as intended is high. The regulatory authorities responsible for the registration of antimicrobial pesticides or biocidal active substances in the United States and Europe (EPA and ECHA, respectively) have concluded that the testing databases supporting ADBAC and DDAC are adequate for risk assessment and that neither of these substances is considered a reproductive toxicant. To avoid waste of limited resources, future research on these

substances must clearly justify dose selection and consider the limited human exposure to these substances.

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CONFLICT OF INTEREST

Keith A. Hostetler, Louan C. Fisher, and Benjamin L. Burruss are consultants to the ADBAC and DDAC Issues Steering Committees, the studies' sponsor. Louan C. Fisher was employed at and participated in the collection and tabulation of the data for these studies conducted by Bushy Run Research, which received funding from the ADBAC and DDAC Issues Steering Committees to conduct these studies.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ENDNOTE

[1] The alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) Issues Steering Committees (ISCs) operate under the auspices of the Household and Commercial Products Association. Member companies are Lonza, Inc.; Mason Chemical, a subsidiary of Pilot Chemical; and Stepan Company. SafeBridge® Regulatory & Life Sciences Group (formerly Toxicology Regulatory Services) has provided toxicology services related to these substances for more than 25 years and is under contract by the ADBAC and DDAC ISCs and the Household and Commercial Products Association.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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