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# Evaluating the male and female reproductive toxicity of high-boiling petroleum substances

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ABSTRACT

To meet the EPA HPV Chemical Challenge Program requirement for reproductive toxicity data on sponsored high-boiling petroleum substances (HBPS), an analysis was conducted using the results of 39 repeat-dose and 59 developmental rat dermal toxicity studies on HBPS samples spanning the boiling range of the sponsored substances, and the results of three one-generation reproductive toxicity studies on two samples spanning the concentration range of polycyclic aromatic compounds of sponsored substances. The analysis found little evidence of male or female reproductive tract toxicity based on histopathology, reproductive organ weight, and sperm parameters, and no evidence of effects on fertility, while significant developmental toxicity and/or systemic repeat-dose toxicity were frequently observed. Among 14 samples of HBPS tested in both repeat-dose toxicity and developmental toxicity studies, there were no studies in which an adverse reproductive tract finding occurred at a dose lower than that producing developmental toxicity or other adverse effects in repeat-dose toxicity studies. The current analysis supports the hypothesis that effects in developmental and/or repeat-dose toxicity studies of HBPS occur at doses lower than those that might affect fertility in rat one-generation reproductive studies. When adequate developmental and repeat-dose toxicity studies are available, a reproductive toxicity study of HBPS appears unnecessary.



## 1. Introduction

A large number of high-boiling petroleum substances (HBPS) i.e., those with final boiling points ⩾ approximately 650 °F (343 °C), are produced in refineries and exist primarily as refinery intermediate and blending streams that are used to produce commercial and industrial products including fuels and lubricants. Categories of HBPS include aromatic extracts, asphalt, crude oil, gas oils, heavy fuel oils, lubricating oil basestocks, waxes and related materials and certain petroleum waste substances (Gray et al., 2013).

HBPS were included in the voluntary high production volume (HPV) Challenge Program due to their high US production or importation levels. This program was announced in 1998 by the US Environmental Protection Agency (US EPA), and it was intended to make available to the agency and the public basic physical–chemical, environmental fate, ecotoxicity and mammalian toxicity data for HPV Chemicals. The number of HBPS sponsored by the Petroleum HPV Testing Group(PHPVTG), as defined by chemical abstract registry number (CASRN), is approximately 110. The composition of HBPS can vary substantially among substances assigned the same CASRN. As such, it is not desirable or feasible to conduct costly animal testing on the large number of potential substances. Samples of only a few HBPS have been tested in reproductive toxicity studies. Because there are so many HBPS, the approach which the petroleum industry has taken traditionally is to test examples of these substances and to use the results to read-across and characterize the hazards of similar substances.

Depending on their refining history, some HBPS may contain various levels of polycyclic aromatic compounds (PAC) (Gray et al., 2013). A few individual polycyclic aromatic hydrocarbons (PAHs), a subclass of polycyclic aromatic compounds (PACs) that may be present at low concentrations relative to alkylated PACs and other components in certain HBPS, have been evaluated for reproductive toxicity in animal studies. In general, these studies

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show that some PAHs in pure form, such as benzo[a]pyrene (BaP), have the potential to cause male and female reproductive toxicity at doses $\geqslant 1$ mg/kg$_{bw}$/day. For example, fertility was significantly reduced among both the male and female offspring of pregnant mice exposed to BaP at oral doses of $\geqslant 10$ mg/kg$_{bw}$/day on gestation days (GD) 7–16 (Mackenzie and Angevine, 1981). In adult male rats, inhalation of BaP at levels $\geqslant 75$ μg/m$^3$ for 4 h/day for 10 days inhibited testicular steroidogenesis, epididymal function and sperm motility; plasma testosterone levels also were significantly decreased compared to controls (Inyang et al., 2003). Similarly, BaP caused a significant decrease in testicular weight, sperm count and motility in rats exposed by inhalation to 75 μg/m$^3$ of BaP for 4 h daily for 60 days (Ramesh et al., 2008). Effects on sperm count, morphology, and motility were reported in a multi-generation fertility study of BaP administered orally to male mice at doses $\geqslant 1$ mg/kg$_{bw}$/day; in this study, female mice were not exposed to BaP (Mohamed et al., 2010).

BaP and other PACs have also been reported to affect female fertility. BaP inhibited ovulation in mice given a single intra-peritoneal injection; doses ranging from 1 to 500 mg/kg$_{bw}$ produced a dose- and time-dependent decrease in the number of corpora lutea (Swartz and Mattison, 1985). Another study in mice suggested that oral administration of 10 mg/kg$_{bw}$/day of BaP suppressed the development of primordial oocytes during fetal development (Kristensen et al., 1995). Three PAHs (BaP, 3-methylcholanthrene, and 9, 10-dimethylbenzanthracene) produced ovotoxicity in rats and mice given repeated intraperitoneal doses for 15 days (Borman et al., 2000). BaP, as well as unalkylated nitrogen-containing PACs, carbazole and benzo[a]carbazole, have been reported to cause developmental toxicity among pregnant rats given oral or intraperitoneal doses of these substances (Archibong et al., 2002; Bui et al., 1986; Dutson et al., 1997; Rigdon and Rennels, 1964).

US EPA provided guidance for fulfilling the reproductive toxicity data requirement under the high production volume (HPV) Challenge Program by adopting the Organization for Economic Cooperation and development (OECD) guidance for meeting the screening information data sets (SIDS) requirements (US EPA, 2013). The guidance includes a variety of combinations of testing requirements depending upon the type of test data already available. In essence, in the absence of adequate animal test data for assessing reproductive toxicity, screening-level tests defined by such guideline protocols as the OECD 421 or 422, a combination 90-day repeat-dose and developmental toxicity studies defined by such guideline protocols as OECD 411 and 414, or a one- or two-generation study defined by such guideline protocols as OECD test guideline 415–416 would be required (OECD, 2012). However, US EPA (2013) states, "when a 90-day repeated dose study is available and is sufficiently documented with respect to studying effects on the reproductive organs and a developmental study is available, the requirements for the reproduction toxicity endpoint are satisfied."

The current analysis grew out of a project initiated to investigate the potential relationship between the PAC content and the acute, repeat-dose, developmental, reproductive and genetic toxicities of HBPS. Early in the project the authors noted that whereas reduced fetal growth and survival was evident in many developmental studies of HBPS, reproductive toxicity, as determined by effects on male and female reproductive organs in repeat-dose studies or by decreased female fertility in developmental studies that began exposure as early as 7 days prior to mating, did not appear to be a sensitive toxicity endpoint for HBPS. Nearly all of the repeat-dose toxicity studies assessed both male and female reproductive organ weights and histopathology and many included a sperm evaluation.

The current investigation was undertaken to meet the HPV Challenge Program requirement for reproductive toxicity data on sponsored HBPS and to test the hypothesis that, in a large series of unpublished studies of HPBS, effects on male and female reproductive organs in repeat-dose toxicity studies are not sensitive indicators of toxicity compared to developmental toxicity and systemic effects observed in repeat-dose toxicity studies. This evaluation included the results of all unpublished repeat-dose, developmental, and reproductive toxicity studies of HBPS administered dermally to rats made available by members of the petroleum HPV testing group (PHPVTG).

## 2. Materials and methods

Member companies of the PHPVTG were asked to provide original laboratory reports for reproductive, developmental and repeat-dose toxicity studies of petroleum substances conducted on samples with corresponding PAC compositional data. These studies which were undertaken primarily for the purpose of product stewardship, not regulatory submission, were conducted in several different laboratories by a number of sponsors over an extended period of time. Most of these studies were conducted in the 1980s and 1990s, although some studies were conducted more recently. All studies were conducted according to good laboratory practices (GLP) or in the spirit of GLPs (US EPA, 2011).

All of the submitted developmental studies were conducted in rats and used dermal application as the route of administration since dermal contact is regarded to be the most likely route of human exposure. Of the repeat-dose toxicity studies most had been carried out in rats exposed via the dermal route. One exception was a target organ toxicity study in male mice (Sample 86001); this 10-week study with interim sacrifices included four groups that had been treated via the oral route and one group via the dermal route. The only other exception was a 13-week dermal study in rats (Sample 86187) that included two groups (males) that had been exposed orally and four groups (males and females) that had been exposed dermally. Only data from the dermally exposed rats were used in this evaluation.

The exact method of dermal application varied among the studies. In all studies, the site of dermal application was shaved. The dermal application of the test material was not occluded in most studies, but it was occluded in a small percentage of studies. The test material was left in place for 6 h in some studies and 24 h in others.

The substances included in the investigation were, as a part of the petroleum industry's HPV program, assigned to the following HPV substance categories: aromatic extracts, asphalt, crude oil, gas oils, heavy fuel oils, lubricating oil basestocks, waxes and related materials, and reclaimed petroleum hydrocarbons (i.e., residual hydrocarbon wastes from petroleum refining).

Fifty-nine developmental toxicity studies of HBPS in Sprague–Dawley rats were provided by the PHPVTG member companies. All of the developmental toxicity studies, which were conducted between the years approximately 1986–2011, used dermal application, the likely route of human exposure, as the route of administration. Most of the developmental toxicity studies are best described as screening studies in which the group size was typically in the range of 10–20 mated females. Fewer than half were full developmental toxicity studies with group sizes of 20 or more mated females. All the studies had at least one concurrent control group, and fifty-three had at least three dose groups. Exposures to the test substance began on either gestation day (GD) 0 or pre-mating day (PMD) 7. All the reports were evaluated and given Klimisch reliability scores of either 1 (reliable without restrictions) or 2 (reliable with restrictions) (Klimisch et al., 1997). No studies were excluded for reasons of reliability or data quality.

PHPVTG member companies also submitted thirty-nine repeat-dose toxicity studies of HBPS performed in rats that included evaluation of reproductive organ weights and/or reproductive organ histopathology. Of these, there were twenty-nine 90-day studies and ten 28-day studies. All of the repeat-dose toxicity studies included both male and female rats, except for one 90-day study in males only (Sample 87058) and one 90-day study in females only (Sample 87293). Thus, there were equal numbers (38) of repeat-dose toxicity studies in males and females. All of the repeat-dose toxicity studies, which were conducted between the years approximately 1982–2011, were carried out via the dermal route and included 5 days/week treatments. The studies were specifically assessed for evidence of reproductive toxicity. The repeat-dose toxicity studies assessed reproductive organ weights and histopathology. Seventeen 90-day studies also assessed sperm quality. In all seventeen studies, the sperm evaluation included an assessment of sperm morphology; sperm count was assessed in fourteen of these studies. The methods employed to evaluate sperm were typical of those used in the 1980s and 1990s. Each of the studies included one concurrent control group, and most included three dosed groups. All the reports were judged to be either "reliable without restrictions" or "reliable with restrictions, i.e., Klimisch reliability scores of 1 or 2 (Klimisch et al., 1997).

The testes and ovaries were evaluated histologically in all of the repeat-dose toxicity studies; in addition, the epididymis, prostate and uterus were evaluated histologically in many of these studies. In most studies, sections of selected tissues, including reproductive organs, were collected and preserved with 10% neutral buffered formalin, embedded in paraffin wax and subsequently stained with hematoxylin and eosin. Five 90-day studies of HBPS (Samples 60901, 10929, 20906, 120801, and 10903), which were conducted more recently (2009–2011), used Bouin's fluid rather than formalin to avoid shrinkage artifacts associated with formalin fixation.

A semen evaluation was performed in seventeen of the 90-day repeat-dose toxicity studies. In most cases, sperm evaluations were performed on ten male rats per group from the control and the highest dose group that survived to the end of the study. In all but one of the seventeen studies, the sperm evaluation was performed at the study's terminal necropsy. The exception was a study of a heavy fuel oil (Sample 86484) in which the sperm evaluation was conducted at week 9 when the high dose group was terminated due to excessive mortality and morbidity. In most cases (14 of 17 studies), the sperm evaluation included a determination of sperm counts in both the testes and caudal epididymis. For the testes, the sperm (spermatid) counts were expressed as millions of sperm per testes and per gram of testes. Similarly, the epididymal sperm (spermatozoa) counts were expressed as millions of sperm per caudal epididymis and per gram of caudal epididymis. Sperm morphology was evaluated in all 17 of the studies in which sperm evaluations were conducted. Sperm motility was assessed in only one study (i.e., Sample 86272), and no effect was found.

In addition to the repeat-dose and developmental toxicity studies described above, three single-generation reproductive toxicity studies on two samples of HBPS were made available to the authors. The design of these studies and the findings from them are described in detail in Section 3.1.

## 3. Results

### 3.1. Reproductive toxicity studies of HBPS

The reproductive toxicity studies of HBPS included the following: (1) a one-generation reproduction study in rats of a white mineral oil (Sample 85018), with little, if any, PAC content, and (2) separate male and female one-generation reproductive toxicity screening studies in rats of a heavy fuel oil refinery stream (Sample F-179), composed primarily of PACs. These studies are summarized below.

#### 3.1.1. White mineral oil (Sample 85018)

An unpublished one-generation reproductive toxicity study was performed on a white mineral oil (Sample 85018; Stock 461; CAS-RN 8042-47-5), a substance which contains little, if any, PACs. Groups of Sprague–Dawley rats (20/sex/dose) received dermal applications of undiluted Sample 85018 of 0 (untreated), 0 (sham-treated), 125, 500, or 2000 mg/kg$_{bw}$/day. Doses were administered for approximately 10 weeks during premating, 3 weeks during the mating period, the 3 weeks of gestation, and 3 weeks postpartum. Dams were sacrificed on day 21 of lactation. The frequency of dosing for females was 5 days/week during premating and mating, daily on GDs 0–20 and 5 days/week during the postpartum period. Males were split into two subgroups within each dose group. Ten of the males in each dose group were dosed 5 days/week during premating and mating. The other ten males per dose group were dosed 5 days/week during premating, mating, and postmating until sacrifice.

The experimental design of this study was similar to OECD guideline 415. Differences included the use of 2000 mg/kg$_{bw}$/day rather than the limit dose of 1000 mg/kg$_{bw}$/day and administration of doses 5 times/week during much of the study rather than 7 times/week. Also, the testing laboratory was closed before microscopic evaluation of tissues was performed and a histopathology report was not prepared.

During gestation and lactation, erythema, scabs, and flaking were observed on the skin of nearly all animals treated with Sample 85018. Body weight gain during the gestation and postpartum periods appeared normal in all dosed groups. Mean body weights of females in the 2000 mg/kg$_{bw}$/day group were significantly lower than the untreated controls during the first half of gestation, but were similar to mean weights for the sham-exposed controls.

No evidence of reproductive toxicity was observed in this study. The estrus cycle, examined prior to and during mating, was not affected by treatment. No effects were seen in the percentage of pregnant females, duration of gestation, or number of implantation sites per dam. No adverse effects were noted on the number of live-born pups or day 4 or day 21 survival indices. Mean pup weight during postnatal days 0–28 was not affected by treatment. The study authors concluded that dermal application of Sample 85018 at doses up to 2000 mg/kg$_{bw}$/day beginning 10 weeks before mating did not have any adverse effects on reproductive performance of male or female rats or on the *in utero* and postnatal survival or development of offspring.

#### 3.1.2. Heavy fuel oil blending refinery stream (Sample F-179)

Two one-generation reproductive toxicity screening studies of a heavy fuel oil blending stream (CASRN 64741-62-4; clarified oils (petroleum), catalytic cracked; Sample F-179 also termed clarified slurry oil or carbon black oil) were conducted, one each in male and female rats (Hoberman et al., 1995). Clarified slurry oil, obtained from the fluidized catalytic cracker unit in a petroleum refinery, is a complex mixture of individual chemical substances. Clarified slurry oils typically contain very high levels of PACs (this sample had about 67% of DMSO-extractable aromatic ring structures which includes PACs) and have been found to be one of the most mutagenic and dermally carcinogenic substances produced by petroleum refining with a high degree of developmental toxicity (i.e., a dermal developmental toxicity lowest observed adverse effect level (LOAEL) of 1 mg/kg$_{bw}$/day) (Blackburn et al., 1986; Hoberman et al., 1995). Because of the high PAC levels, samples of this substance have historically been considered as "worst case" examples when compared to other HBPS.

In the male reproductive toxicity screening study of Sample F-179, groups of ten male rats were given the test substance dermally at doses of 0, 0.1, 1, 10, 50, and 250 mg/kg$_{bw}$/day. The test material was given for 70 days prior to a seven-day cohabitation period with untreated female rats. There were no signs of skin irritation at any dose. Doses of 10 mg/kg$_{bw}$/day or higher resulted in reduced body weight and food consumption. Administration of 50 and 250 mg/kg$_{bw}$/day caused adverse clinical effects in the male rats. However, mating and fertility parameters were unaffected by doses of up to 250 mg/kg$_{bw}$/day, the highest dose assessed in the study. The test material also had no significant effect on sperm quality, including sperm count, concentration, motility, and morphology. The study authors concluded that the no observed adverse effect level (NOAEL) for fertility and reproductive organ effects in the male rats was greater than 250 mg/kg$_{bw}$/day (the highest dose tested), while the NOEL for general toxicity in the male rats was 1 mg/kg$_{bw}$/day (based on reduced body weight and food consumption observed at 10 mg/kg$_{bw}$/day and higher).

In the female reproductive toxicity screening study of Sample F-179, groups of ten female rats were administered the test material dermally at doses of 0, 0.1, 1, 10, 50, and 250 mg/kg$_{bw}$/day. The test material was given for 14 days prior to a cohabitation period with untreated male rats and continued until day 0 of presumed gestation. In this study, a Caesarean-section was performed on GD 14. Maternal toxicity was observed at doses of 50 mg/kg$_{bw}$/day and greater. Decreased body weight gain was reported at 50 and 250 mg/kg$_{bw}$/day; food consumption was significantly decreased at the 250 mg/kg$_{bw}$/day. Estrous cycling, mating, fertility parameters, and reproductive organ weights were unaffected by administration of the test substance at doses as high as 250 mg/kg$_{bw}$/day. No effect on the offspring was observed at any dose at the time of Caesarean-section. Thus, the study authors concluded that the reproductive NOAEL in the female rats was at least 250 mg/kg$_{bw}$/day (the highest dose tested), and the NOAEL for general toxicity among female rats was 10 mg/kg$_{bw}$/day (based on decreased body weight gain and food consumption at 50 mg/kg$_{bw}$/day and higher).

While the male or female reproductive toxicity screening studies of Sample F-179 differ in some respects from current regulatory guidelines for a reproductive toxicity study, they provide valuable information on the potential reproductive toxicity of a "worst case" heavy fuel oil blending stream. In both males and females, reproductive endpoints were unaffected at 250 mg/kg$_{bw}$/day, the highest dose tested. Importantly, these reproductive toxicity screening studies do not suggest that reproductive toxicity is a sensitive endpoint of toxicity for this sample of clarified slurry oil, particularly when compared with the results of the repeat-dose and developmental toxicity studies of clarified slurry oil, as described in Section 3.4.

#### 3.1.3. Summary of reproductive toxicity studies

The results of the one-generation reproductive toxicity studies of white mineral oil and clarified slurry oil are considered to define the range of outcomes that would be expected for other HBPS because they span the PAC concentration range in HBPS. A white mineral oil (Sample 85018) with little or no PAC produced no evidence of reproductive toxicity at dermal doses up to 2000 mg/kg$_{bw}$/day. Similarly, a heavy fuel oil blending stream (Sample F-179), with high PAC content, produced no effects on male or female fertility in separate male and female reproductive toxicity screening studies in rats given dermal doses up to 250 mg/kg$_{bw}$/day, a dose twenty-five times greater than the dose required to produce clear systemic toxicity (see Section 3.4). While the reproductive toxicity studies of this heavy fuel oil component and the white mineral oil suggest little, if any, potential for male or female reproductive toxicity, the small number of reproductive toxicity studies limits the ability to draw more general conclusions for the large group of HBPS. To further investigate the potential for these substances to pose a reproductive toxicity hazard, repeat-dose toxicity studies of HBPS were reviewed for evidence of effects on reproductive organs.

### 3.2. Evaluation of reproductive organ toxicity in repeat-dose toxicity studies of HBPS

Data from the repeat-dose toxicity studies suggest that HBPS have limited potential to affect male or female reproductive organs or sperm quality. In most studies, no effect on reproductive organs was observed. In the few cases where possible effects on reproductive organs were observed, these occurred only at high doses and often in the presence of high mortality.

#### 3.2.1. Male reproductive organs

*3.2.1.1. Testes, prostate, and epididymis weights.* The testes were weighed in 38 repeat-dose studies of HBPS. There was little evidence of any differences in the absolute or relative weights of the testes between dosed and control groups in these studies. Table 1 summarizes the study results of the observations on male reproductive organ weights. In most cases, the dose presented in Table 1 is the highest dose group in the study. However, in four studies (Samples 83366, 86484, 87213, and 86187), the study had two higher dose level groups which were terminated early (ranging from 2 to 13 weeks) due to excessive mortality and morbidity. The results of the terminated dose groups are not presented in Table 1, either because reproductive organ weights were not evaluated and therefore not available or because changes in reproductive organ weights were considered by the study authors to be secondary to systemic toxicity.

Absolute testes weight was significantly decreased in only one of 38 studies (Sample 8281). The decrease was observed only at the high dose (500 mg/kg$_{bw}$/day), well above the dose that produced systemic toxicity in this study. Relative testes weight, generally a less useful metric than absolute testes weight, was not significantly decreased in any of the 38 studies. In contrast, relative testes weight was significantly increased in seven studies; an increase in relative testes weight is not usually regarded as an adverse reproductive effect. The increases in relative testes weight are attributable to reductions in body weight. Thus, the evidence of an adverse effect on testicular weight was limited to a significant decrease in absolute weight in only one of 38 studies.

The prostate and epididymides were also weighed in twenty-four of these same 38 repeat-dose studies, and the test substances also had little, if any, effect on prostate or epididymis weights. Significant decreases in absolute prostate weights were observed in only three of the 18 studies (Samples 85244, 86271 and 89106) – and only at the high dose in each study (i.e., 2000, 500, and 1000 mg/kg$_{bw}$/day, respectively). Relative prostate weight was not significantly decreased in any of the 24 studies. An increase in relative prostate weight was observed in one study (Sample 87213).

Epididymis weights (absolute) were significantly decreased in three of 24 studies (Samples 86181, 89106, and 10903). Relative epididymis weight was not significantly decreased in any of the 18 studies. A significant increase in relative epididymis weight was observed in two studies (Samples 85244 and 87213) at doses of 2000 and 125 mg/kg$_{bw}$/day, respectively; these same doses were also associated with significant reductions in body weight and significant increases in relative testes, liver, kidney, brain and adrenal weights.

*3.2.1.2. Testicular histopathology.* Histological examination of the testes was performed in 38 repeat-dose toxicity studies of HBPS in rats. Among the 38 repeat-dose studies, there was some evi-

**Table 1**
Summary of male reproductive organ weights in repeat-dose studies of HBPS administered dermally to rats.

| HBPS category | CAS no. | Sample no. | Duration, days | Dose[a], mg/kg$_{bw}$/day | Testes Wt. | | Prostate Wt. | | Epididymis Wt. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Absolute | Relative | Absolute | Relative | Absolute | Relative |
| Heavy fuel oil | 64741-62-4 | 86484 | 90 | 30[a] | – | – | – | – | – | – |
| Heavy fuel oil | 64741-62-4 | 86001 | 90 | 125 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-115 | 28 | 50 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-179 | 90 | 530 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-73-01 | 28 | 2710 | – | ↑[c] | ND | ND | ND | ND |
| Heavy fuel oil | 64741-62-4 | 10929 | 90 | 50 | – | ↑ | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 83366 | 90 | 125[a] | – | – | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 86181 | 90 | 125 | –[d] | – | – | – | ↓[e] | – |
| Heavy fuel oil | 64741–81-7 | 86193 | 90 | 125 | – | – | – | – | – | – |
| Heavy fuel oil | 64741–81-7 | 86272 | 90 | 125 | – | – | – | – | – | – |
| Heavy fuel oil | 64741-80-6 | 86192 | 90 | 1000 | – | – | – | – | – | – |
| Heavy fuel oil | 64741-75-9 | F-127 | 28 | 210 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-57-7 | F-128 | 28 | 2350 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-57-7 | 85244 | 90 | 2000 | – | ↑ | ↓ | – | – | ↑ |
| Heavy fuel oil | 64741-45-3 | F-132 | 28 | 940 | – | – | ND | ND | ND | ND |
| Heavy fuel oil | 64741-61-3 | F-134 | 28 | 990 | – | – | ND | ND | ND | ND |
| Gas oil | 68915-97-9 | 86271 | 90 | 500 | – | – | ↓ | – | – | – |
| Gas oil | 64741-82-8 | 87213 | 90 | 125[a] | – | ↑ | – | ↑ | – | ↑ |
| Gas oil | 64741-49-7 | 86270 | 90 | 500 | – | – | – | – | – | – |
| Gas oil | 64741-59-9 | 8281 | 90 | 500 | ↓ | – | ND[f] | ND | ND | ND |
| Gas oil | 64741-59-9 | 10903 | 90 | 750 | – | ↑ | – | – | ↓ | – |
| Gas oil | 64741-77-1 | F-188 | 28 | 820 | – | – | ND | ND | ND | ND |
| Gas oil | Blend[b] | F-129 | 28 | 235 | – | – | ND | ND | ND | ND |
| Gas oil | 64741-43-1 | F-130 | 28 | 460 | – | – | ND | ND | ND | ND |
| Gas oil | 64741-86-2 | F-233 | 28 | 820 | – | – | ND | ND | ND | ND |
| Gas oil | 68334-30-5 | 120801 | 90 | 600 | – | – | – | – | – | – |
| Crude Oil | 8002-05-9 | 89645 | 90 | 500 | – | – | – | – | – | – |
| Crude Oil | 8002-05-9 | 89646 | 90 | 500 | – | – | – | – | – | – |
| Aromatic extract | 64742-04-7 | 86187 | 90 | 125[a] | – | – | –[g] | – | – | – |
| Aromatic extract | 64742-10-5 | 86525 | 90 | 2000 | – | – | – | – | – | – |
| Aromatic extract | 64742-10-5 | 87058 | 90 | 2000 | – | – | – | – | – | – |
| Aromatic extract | 64742-10-5 | 87476 | 90 | 2000 | – | – | – | – | – | – |
| Aromatic extract | 64742-05-8 | 20906 | 90 | 150 | – | – | – | – | – | – |
| Asphalt | 64741-56-6 | 86268 | 90 | 1000 | – | – | – | – | – | – |
| Petroleum waste | 68477-27-0 | 89106 | 90 | 1000 | – | ↑ | ↓ | – | ↓ | – |
| Lubricating oil basestock[c] | 64742-65-0 | 89040 | 90 | 2000 | – | – | – | – | – | – |
| Lubricating oil basestock[c] | 64742-65-0 | 82191 | 90 | 1720 | – | ↑ | ND | ND | ND | ND |
| Lubricating oil basestock[c] | 64742-65-0 | 60901 | 90 | 1000 | – | – | – | – | – | – |

↓ = Statistically significant decrease; ↑ = statistically significant increase; – = no significant effect; ND = no data.

[a] In most cases, the dose presented in this table is the highest dose group in the study. However, in four studies (Samples 83366, 86484, 87213, and 86187), the study had two higher dose level groups which were terminated early (ranging from 2 to 13 weeks) due to excessive mortality and morbidity; the results of the terminated dose groups are not presented in this table because reproductive organ weights were not evaluated or because changes in reproductive organ weights were considered to be secondary to excessive systemic toxicity.

[b] F-129 is a blend of three gas oils (CASRNs. 64741-58-8, 64741-59-9, 64741-82-8).

[c] A stastically significant (but not dose-related) increase in testes weight relative to body weight was observed at all dose levels (542, 1084, and 2710 mg/kg$_{bw}$/day).

[d] The weight of the testes was not statistically significantly decreased at any dose. However, there was an 8% statistically significant decrease in the weight of testicular parenchyma at this dose; this endpoint was not assessed at lower doses.

[e] A 15% statistically significant decrease in absolute epididymis weight was observed at 30 mg/kg$_{bw}$/day and 125 mg/kg$_{bw}$/day.

[f] At gross necropsy, the prostate was described as "small" in 8/10 males administered 500 mg/kg$_{bw}$/day compared to 1/10 among the controls.

[g] No significant effect on prostate weight was observed; however, histopathological findings in the prostate (i.e., atrophy, cystic glands, prostatitis) were reported in 2/10, 4/10, and 9/10 males administered 0, 30, and 125 mg/kg$_{bw}$/kg, respectively.

dence of an effect on testicular histopathology in only four studies (Samples 86271, 86484, 89106, and 10929), and in all four studies, histological changes in the testes were seen only at doses associated with severe systemic toxicity.

In the first study (Sample 86271), testicular atrophy and the absence of spermatozoa in the epididymis were observed in a single male rat given the high dose of 500 mg/kg$_{bw}$/day, a dose that produced a severe reduction in hematopoiesis in the bone marrow in ten of ten males. Systemic toxicity, including effects on the hematopoietic system, liver and thymus, was seen at $\geqslant$125 mg/kg$_{bw}$/day. The study authors did not consider the testicular histopathology in the one male rat to be treatment-related. No evidence of an effect on testicular histopathology was observed in the other nine high dose males in which testes were examined microscopically. In addition, an additional group of ten male rats given the same high dose of 500 mg/kg$_{bw}$/day for the same duration in the same laboratory were subjected to a semen evaluation, as described in detail in the next section, and there was no evidence of an adverse effect on sperm quality among these rats.

In the second study (Sample 86484), all of the males in the two highest dose groups (i.e., 125 and 500 mg/kg$_{bw}$/day) died or were sacrificed before scheduled necropsy. For these groups, the study authors considered atrophy of the reproductive organs to be a secondary effect of cachexia, debility, and/or moribund condition. At the next lowest dose (i.e., 30 mg/kg$_{bw}$/day), 2 of 10 males died or were sacrificed before scheduled necropsy. At this dose, histological effects in the testes were reported in two males with focal atrophy and two different males with interstitial focal hemorrhage; the testes were reported as normal in the remaining six males. This dose was associated with significant anemia and thrombocytopenia, as well as significant histological changes in the bone marrow, liver, lymph nodes and lungs. At the lowest dose, 8 mg/kg$_{bw}$/day,

which still produced significant systemic toxicity, no evidence of a histological effect in the testes was observed.

In the third study (Sample 89106), congestion, hemorrhage, or both were reported in the testes of 4 of 10 male rats exposed to 1000 mg/kg$_{bw}$/day, a dose associated with mortality and severe histopathological effects in the bone marrow. In the fourth study (Sample 10929), an increased incidence of seminiferous tubular degeneration in the testis and hypospermia and luminal cellular debris in the epididymis was reported at 50 mg/kg$_{bw}$/day, a dose that produced mortality, bone marrow depletion, and other histopathological effects. This was one of the five recent 90-day studies that used state-of-the-art methodology for fixation and examination of the testes, as described earlier, and the only one that demonstrated testicular histopathological effects.

The relative lack of histopathology of the testes at even the high dose level in the repeat-dose toxicity studies when compared to the histologic changes observed in other organs (e.g., liver, thymus, bone marrow) at the same or lower doses provides further evidence that the testes are not a sensitive target of toxicity for HBPS.

*3.2.1.3. Sperm evaluation.* Among the 38 repeat-dose dermal studies of HBPS, sperm evaluations were conducted in 17 studies, all of which were 90-day studies. Sperm evaluations consisted of an assessment of sperm concentrations (in both testes and cauda epididymis), and sperm morphology in 14 and 17 studies, respectively. The results of these sperm evaluations are presented in Table 2. There was one study (Sample 86272 at 125 mg/kg$_{bw}$/day) in which sperm motility was assessed, and no effect was found.

Based on the testicular samples, spermatid concentration was not significantly decreased compared to controls for any of the 14 substances evaluated for this endpoint. In the cauda epididymis, a statistically significant decrease in sperm concentration was observed with three substances (Samples 86484, 86181, and 89106). Two of these (Samples 86484 and 89106) were among those that examined the same types of substances that demonstrated suggestive evidence of testicular histopathology, as described in Section 3.2.1.2. As noted earlier, these effects were seen only at doses associated with severe systemic toxicity, suggesting that these changes were most likely secondary to systemic toxicity rather than direct effects on reproductive functions. For example, with Sample 86484, the sperm evaluation was performed after only 9 weeks of exposure because the high dose group (500 mg/kg$_{bw}$/day) was terminated after 9 weeks due to severe toxicity. No effect on sperm concentration in the testes or epididymis was observed with this substance in rats treated with 30 mg/kg$_{bw}$/day, the highest dose at which animals survived to the end of the study. The results of these 90-day studies indicate that HBPS had little or no effect on sperm concentration.

Sperm morphology was not significantly different from the control group in any of the seventeen studies. The percentage of normal sperm ranged from 95% to 98% for all test substances except for one study (Sample 86484), in which the percentage of normal sperm was 91% at the high dose (not statistically significantly different from the control value).

In summary, based on a sample of seventeen 90-day dermal studies in rats of HBPS, there is limited evidence of an adverse effect on sperm quality. In most cases, the sperm evaluation was conducted on the highest dose group surviving to the terminal necropsy. Thus, spermatogenesis does not appear to be a sensitive target of toxicity for these materials when administered dermally

*3.2.2. Female reproductive organs*

*3.2.2.1. Ovarian and uterine weights.* The ovaries were weighed in 38 repeat-dose toxicity studies. The uterus was weighed in a subset of 24 repeat-dose toxicity studies, all of which were 90-day studies. As seen in Table 3, HBPS demonstrated minimal potential to alter the weights of the ovaries and uterus.

The absolute weight of the ovaries was significantly decreased in 4 of 38 studies (Samples F-233, 86001, F-115, F-73-01). In one study (Sample 86001), both the absolute and relative weights of the ovaries were significantly decreased at 125 mg/kg$_{bw}$/day, a dose associated with 40% mortality. A significant increase in relative weight of the ovaries was observed in one study (Sample 8281) at 500 mg/kg$_{bw}$/day likely due to reduced body weight rather than increased absolute ovarian weight; females in this group weighed about 7% less than controls at the end of the study and exhibited severe skin reactions including erythema, edema and stiff leathery skin. There were no other statistically significant differences in absolute or relative weight of the ovaries in any of the other studies.

Uterine weights were available from 24 repeat-dose toxicity studies. A significant decrease in absolute, but not relative, uterine weight was observed at the high dose in one repeat-dose toxicity study (Sample 10903). In another study (Sample 85244), a statistically significant increase in relative, but not absolute, uterine weight was observed at the high dose, which was associated with a significant decrease in body weight. No statistically significant decrease or increase in absolute or relative uterine weight was reported in any other study.

*3.2.2.2. Ovarian histopathology.* The histopathology of the ovaries was evaluated in the 38 repeat-dose toxicity studies for which ovarian and uterine weights were recorded (see Section 3.2.2.1). Based on these studies, pathologic changes in ovarian histology is not a sensitive outcome in studies of HBPS. Decreased corpora lutea, increased atretic follicles, and atrophy of the uterus with increased prominence of stromal cells were reported in one repeat-dose toxicity study (Sample 10929) at the high dose level, which also produced mortality and systemic toxicity, including bone marrow depletion. "Possible hypoplasia" of the ovaries at gross necropsy was reported in another repeat-dose toxicity study (Sample 86001) at a dose associated with 40% mortality. No compound-related adverse effects on ovarian histology were reported in any other study.

In summary, there was little evidence of male or female reproductive toxicity in a large number of repeat-dose toxicity studies of HBPS. In the few cases where possible effects on reproductive organs were observed, these occurred only at high doses and often in the presence of high mortality. In these studies, reproductive organ weights and histopathology were not among the most sensitive endpoints of toxicity.

### *3.3. Evidence of reproductive toxicity in developmental toxicity studies of HBPS*

*3.3.1. Decreased fetal/pup survival and growth*

A review of the developmental toxicity studies of HBPS demonstrates that decreased fetal and pup survival and growth are the most sensitive indicators of developmental toxicity for these substances. For the current project, 59 unpublished laboratory reports of developmental toxicity studies of HBPS administered dermally were reviewed. Twenty-nine of the developmental toxicity studies were of a traditional design in which the pregnant rats were exposed during gestation, and the uterine contents were examined during a c-section just prior to birth. These studies were called "Type I" developmental toxicity studies. In the remaining thirty developmental toxicity studies, pregnant rats were exposed during gestation, litters were allowed to be delivered naturally, and the offspring were observed from the day of birth through postnatal day 4 (PND 4) or in two studies to PND 28. These studies were termed "Type II" developmental toxicity studies.

**Table 2**
Summary of sperm evaluations among 90-day studies of HBPS Administered dermally to rats.

| HBPS category | CAS no. | Sample no. | Dose[a], mg/kg$_{bw}$/day | Testicular spermatid count | | Epididymal sperm count | | Epididymal sperm morphology (% normal) |
|---|---|---|---|---|---|---|---|---|
| | | | | Per testes | Per g of testes | Per cauda E. | Per g of cauda E. | |
| Heavy fuel oil | 64741-62-4 | 86484 | 500[b] | – | – | ↓ | ↓ | – |
| Heavy fuel oil | 64741-81-7 | 86193 | 125 | – | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 86181 | 125 | – | – | ↓ | – | – |
| Heavy fuel oil | 64741-81-7 | 86272 | 125 | – | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 83366 | 125[a] | ND | ND | ND | ND | – |
| Heavy fuel oil | 64741-80-6 | 86192 | 1000 | – | – | – | – | – |
| Heavy fuel oil | 64741-57-7 | 85244 | 2000 | ND | ND | ND | ND | – |
| Gas oil | 68915-97-9 | 86271 | 500 | – | – | – | – | – |
| Gas oil | 64741-49-7 | 86270 | 500 | – | – | – | – | – |
| Gas oil | 64741-82-8 | 87213 | 125[a] | – | – | – | – | – |
| Crude oil | 8002-05-9 | 89645 | 500 | – | – | – | – | – |
| Crude oil | 8002-05-9 | 89646 | 500 | – | – | – | – | – |
| Aromatic extract | 64742-10-5 | 87476 | 2000 | – | – | – | – | – |
| Aromatic extract | 64742-10-5 | 86525 | 2000 | – | – | – | – | – |
| Aromatic extract | 64742-04-7 | 86187 | 125[a] | ND | ND | ND | ND | – |
| Asphalt | 64741-56-6 | 86268 | 1000 | – | – | – | – | – |
| Petroleum waste | 68477-27-0 | 89106 | 1000 | – | – | ↓ | – | – |

↓ = statistically significant decrease; ↑ = statistically significant increase; – = no significant effect; ND = no data.
[a] The results in this table represent the highest dose level in which sperm evaluations were performed. Samples 83366, 87213, and 86187 had two higher dose level groups which were terminated early (ranging from 2 to 13 weeks) due to excessive mortality and morbidity; no sperm evaluation was performed on these early termination groups.
[b] The high dose (500 mg/kg$_{bw}$/day) in this study (Sample. 86484) was terminated at 9 weeks due to excessive mortality and morbidity. The results of the sperm evaluation at week 9 are presented in this table.

Tables 4 and 5 summarize the LOAELs and the key effects observed at the LOAELs among the Type I and II developmental toxicity studies, respectively. Among the Type I studies, the effects most commonly observed at the LOAELs included decreased number of live fetuses per litter, increased percentage of resorptions, and decreased fetal body weights. Similarly, among the Type II studies, decreases in the number of live pups at birth and in pup body weight at birth were among the endpoints most frequently affected.

Clear evidence of developmental toxicity was observed in most developmental toxicity studies of HBPS. In general, substances with a high PAC content, such as Samples 86484 and 86001, exhibited significant developmental toxicity at dermal doses in the range of 1–30 mg/kg$_{bw}$/day. In comparison, studies of the HBPS with low PAC content (<1%), such as Sample 060901, often showed no evidence of developmental toxicity even at dermal doses of 1000–2000 mg/kg$_{bw}$/day. Effects observed less frequently in the developmental toxicity studies included a decrease in the mean number of uterine implantation sites and delayed parturition. Because these endpoints are potentially evidence of female reproductive toxicity, they are evaluated in detail below.

### 3.3.2. Decreased implantation sites

Evidence of a decrease in the mean number of implantation sites per dam was observed in 6 of 30 Type II developmental toxicity studies (Table 6). The possibility exists that this may represent an effect on female reproductive capacity. However, it is also possible that the increase in pre-implantation loss is due to a lethal effect on the conceptus since, in the Type I developmental toxicity studies, post-implantation loss (i.e., an increase in resorptions or dead embryos after implantation) was one of the most sensitive endpoints.

Implantation usually occurs around GD 6 in rats. In all six studies, dosing included GD 0–6. The dosing period started on pre-mating day 7 (PMD 7) in 4 of these studies and continued beyond implantation. In the other two studies, the dosing period began on GD 0 and continued past the day of implantation. Given the timing of the dosing period, it is biologically plausible that the test materials caused a decrease in the mean number of implantations per dam.

The magnitude of the decrease in the mean number of implantations per dam is noteworthy. The decreases, ranging from 6% to 27% in these six studies, indicate the dams were able to become pregnant and implant most of their concepti. If the decreases were due to an inhospitable uterine environment, it does not appear to have been so inhospitable as to prevent most of the concepti from implanting in the uterus. Conversely, if the decreases were due to a direct lethal effect of the test materials on the concepti, the materials did not kill more than 27% of the concepti prior to implantation in any dose group.

Pre-implantation loss occurs in untreated as well as treated rodents and contributes to the normal variation in litter size. After mating, uterine and oviductal contractions are critical in the transport of spermatozoa from the vagina. In rodents, sufficient stimu-

**Table 3**
Summary of female reproductive organ weights in repeat-dose studies of HBPS administered dermally to rats.

| HBPS category | CAS no. | Sample no. | Duration, days | Dose[a], mg/kg$_{bw}$/day | Ovary Wt. | | Uterus Wt. | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Absolute | Relative | Absolute | Relative |
| Heavy fuel oil | 64741-62-4 | 86484 | 90 | >30 | – | – | – | – |
| Heavy fuel oil | 64741-62-4 | 86001 | 90 | 125 | ↓ | ↓ | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-115 | 28 | 50 | ↓ | – | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-179 | 90 | 530 | – | – | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-73-01 | 28 | 1084 | ↓ | – | ND | ND |
| Heavy fuel oil | 64741-62-4 | 10929 | 90 | 50 | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 83366 | 90 | 125 | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 86181 | 90 | 125 | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 86193 | 90 | 125 | – | – | – | – |
| Heavy fuel oil | 64741-81-7 | 86272 | 90 | 125 | – | – | – | – |
| Heavy fuel oil | 64741-57-7 | 85244 | 90 | 2000 | – | – | – | ↑ |
| Heavy fuel oil | 64741-80-6 | 86192 | 90 | 1000 | – | – | – | – |
| Heavy fuel oil | 64741-75-9 | F-127 | 28 | 210 | – | – | ND | ND |
| Heavy fuel oil | 64741-57-7 | F-128 | 28 | 2350 | – | – | ND | ND |
| Heavy fuel oil | 64741-45-3 | F-132 | 28 | 940 | – | – | ND | ND |
| Heavy fuel oil | 64741-61-3 | F-134 | 28 | 990 | – | – | ND | ND |
| Gas oil | 68915-97-9 | 86271 | 90 | 500 | – | – | – | – |
| Gas oil | 64741-82-8 | 87213 | 90 | 125 | – | – | – | – |
| Gas oil | 64741-49-7 | 86270 | 90 | 500 | – | – | – | – |
| Gas oil | 64741-59-9 | 8281 | 90 | 500 | – | ↑ | ND | ND |
| Gas oil | 64741-59-9 | 10903 | 90 | 750 | – | – | ↓ | – |
| Gas oil | 64741-77-1 | F-188 | 28 | 820 | – | – | ND | ND |
| Gas oil | Blend[b] | F-129 | 28 | 235 | – | – | ND | ND |
| Gas oil | 64741-43-1 | F-130 | 28 | 460 | – | – | ND | ND |
| Gas oil | 64741-86-2 | F-233 | 28 | 820 | ↓ | – | ND | ND |
| Gas oil | 68334-30-5 | 120801 | 90 | 600 | – | – | – | – |
| Crude oil | 8002-05-9 | 89645 | 90 | 500 | – | – | – | – |
| Crude oil | 8002-05-9 | 89646 | 90 | 500 | – | – | – | – |
| Aromatic extract | 64742-04-7 | 86187 | 90 | 500 | – | – | – | – |
| Aromatic extract | 64742-10-5 | 86525 | 90 | 2000 | – | – | – | – |
| Aromatic extract | 64742-10-5 | 87476 | 90 | 2000 | – | – | – | – |
| Aromatic extract | 64742-10-5 | 87293 | 90 | 2000 | – | – | – | – |
| Aromatic extract | 64742-05-8 | 20906 | 90 | 150 | – | – | – | – |
| Asphalt | 64741-56-6 | 86268 | 90 | 1000 | – | – | – | – |
| Petroleum waste | 68477-27-0 | 89106 | 90 | 1000 | – | – | – | – |
| Lubricating oil basestock | 64742-65-0 | 89040 | 90 | 2000 | – | – | – | – |
| Lubricating oil basestock | 64742-65-0 | 82191 | 90 | 1720 | – | – | ND | ND |
| Lubricating oil basestock | 64742-65-0 | 60901 | 90 | 1000 | – | – | – | – |

↓ = Statistically significant decrease; ↑ = statistically significant increase; – = no significant effect; ND = no data.
[a] Dose is highest dose evaluated for female reproductive organ weights. In most cases, it is the high dose group in the study.
[b] F-129 is a blend of three gas oils (CASRNs. 64741-58-8, 64741-59-9, 64741-82-8).

lation during mating is necessary for initiation of those contractions. Thus, impaired mating behavior may affect sperm transport and fertilization rate. However, the increase in pre-implantation loss occurred in two studies (i.e., Samples F-222 and F-274) in which the dosing did not begin until GD 0, i.e., after mating. Consequently, in these two studies, it is not possible that the test materials interfered with mating.

In none of these six studies was the decrease in implantation sites a more sensitive effect than other developmental toxicity effects observed in these studies, as shown by the LOAELs for overall developmental toxicity in the last column of Table 6. Other adverse developmental effects were seen at lower doses (4 of 6 studies) or the same dose (2 of 6 studies) compared to the dose associated with a decrease in implantation sites. Whether the increase in pre-implantation loss is a female reproductive effect or a direct lethal effect on the conceptus, it is not a more sensitive toxic endpoint than other endpoints (i.e., increased post-implantation loss and reduced pup body weight at birth) observed in the developmental toxicity studies.

#### 3.3.3. Delayed parturition

Delayed parturition, as measured by a statistically significant increase in the average length of gestation, was observed in seven of the 30 Type II developmental toxicity studies (Table 7). The magnitude of the statistically significant increases in the length of gestation relative to the control values ranged from 0.4 to 0.8 days, increases of approximately 2–4%.

Delayed parturition was not a more sensitive endpoint than other effects observed in the developmental toxicity studies. In all seven studies, the dose(s) that produced delayed parturition also produced a significant reduction in litter size, pup body weight at birth, or both. Reduced litter size and pup birth weight have been shown to be associated with delayed parturition (Marty et al., 2009).

It is also possible that the delayed parturition may be related to non-specific maternal toxicity. Delayed parturition was associated with decreased maternal body weight in 6 of the 7 studies in Table 7. However, a similar effect on maternal body weight was observed with other studies of HBPS, and no effect on gestation length was seen. Thus, there is no consistent evidence that delayed parturition is secondary to general maternal toxicity.

### 3.4. Integrated evaluation of reproductive toxicity observed in reproductive, repeat-dose, and developmental toxicity studies of HBPS

#### 3.4.1. Comparison of results of developmental, repeat-dose, and reproductive screening toxicity studies of clarified slurry oil

One substance, i.e., clarified slurry oil (Samples F179 and 86001), was evaluated in three types of studies: one-generation reproductive toxicity screening, 90-day repeat-dose toxicity and

**Table 4**
Summary of developmental toxicity LOAELs and key effects observed at the LOAEL in developmental toxicity studies (Type I)[a] of HBPS administered dermally to rats.

| HBPS category | CAS no. | Sample no. | Dosing days | Dev. Tox. LOAEL, mg/kg$_{bw}$/day | Percent pregnant | Percent pre-implantation loss | No. of live fetuses per litter | Percent resorptions | Fetal body weight |
|---|---|---|---|---|---|---|---|---|---|
| Heavy fuel oil | 64741-62-4 | 86484 | GD 0–19 | 8 | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 64741-62-4 | 86001 | GD 0–19 | 30 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-62-4 | F-115 | GD 0–19 | 1 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-62-4 | F-179 | GD 0–19 | >0.05[b] | – | – | – | – | – |
| Heavy fuel oil | 64741-62-4 | 10929 | GD 0–19 | 25 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-81-7 | 83366 | GD 0–19 | 125 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-81-7 | 86181 | GD 0–19 | 125 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-81-7 | 86193 | GD 0–19 | >250[e] | – | – | – | – | – |
| Heavy fuel oil | 64741-57-7 | 85244 | GD 0–19 | 500 | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-57-7 | F-196 | GD 0–19 | 150[d] | – | – | ↓ | ↑ | ↓ |
| Heavy fuel oil | 64741-57-7 | F-197 | GD 0–19 | 250 | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 68410-00-4 | F-215 | GD 0–19 | >500[e] | – | – | – | – | – |
| Gas oil | 68915-97-9 | 86271 | GD 0–19 | 125 | – | – | – | – | ↓[c] |
| Gas oil | 64741-82-8 | 87213 | GD 0–15 | >250[f] | – | – | – | – | – |
| Gas oil | 64741-49-7 | 86270 | GD 0–19 | 500 | – | – | ↓ | ↑ | ↓ |
| Gas oil | 64741-59-9 | 8281 | GD 0–15 | 1000 | – | – | ↓ | ↑ | ↓ |
| Gas oil | 64741-59-9 | 10913 | GD 0–19 | 450 | – | – | ↓ | ↑ | ↓ |
| Gas oil | 64741-43-1 | F-193 | GD 0–19 | 250 | – | – | ↓ | – | ↓ |
| Gas oil | 68334-30-5 | F-195 | GD 0–19 | >300[e] | – | – | – | – | – |
| Gas oil | 68334-30-5 | 120801 | GD 0–19 | >600[e] | – | – | – | – | – |
| Gas oil | 64741-82-8 | F-199 | GD 6–11 | >250[e] | – | – | – | – | – |
| Crude oil | 8002-05-9 | 89645 | GD 0–19 | 2000 | – | – | ↓ | ↑ | ↓ |
| Crude oil | 8002-05-9 | 89646 | GD 0–19 | 500 | – | – | – | ↑ | ↓ |
| Aromatic extract | 64742-04-7 | 86187 | GD 0–19 | 125 | – | – | ↓ | ↑ | ↓ |
| Aromatic extract | 64742-10-5 | 87476 | GD 0–19 | >2000 | – | – | – | – | – |
| Aromatic extract | 64742-05-8 | 20906 | GD 0–19 | 25 | – | – | – | – | ↓ |
| Petroleum waste | 68477-27-0 | 89106 | GD 0–19 | 500 | – | – | ↓ | ↑ | ↓ |
| Lubricating oil basestock | 64742-65-0 | 60901 | GD 0–19 | >1000[e] | – | – | – | – | – |
| Lubricating oil basestock | 64742-65-0 | 89040 | GD 0–19 | >2000 | – | – | – | – | – |

↓ = Statistically significant decrease; ↑ = statistically significant increase; –= no significant effect; ND = no data.

[a] Type I developmental toxicity studies are defined as developmental toxicity studies of traditional design in which the pregnant rats were exposed during gestation and the uterine contents were examined at c-section just prior to birth.

[b] The only dose of F-179 tested on GD 0–19 was 0.05 mg/kg$_{bw}$/day. Administration of 250 mg/kg$_{bw}$/day of F-179 on GD 6–8 increased resorptions.

[c] Statistically significant decrease in fetal body weight among males but not among females.

[d] Increases in skeletal variants reported at 75 mg/kg$_{bw}$/day and higher. Decreased live pups per litter, increased resorptions, and decreased fetal body weight were observed at doses of 150 mg/kg$_{bw}$/day.

[e] Highest dose tested; no developmental toxicity observed.

[f] No treatment-related developmental toxicity was observed at 60 mg/kg$_{bw}$/day on GD 1–19, 250 mg/kg$_{bw}$/day on GD 1–15 or at 500 mg/kg$_{bw}$/day on GD 10–12.

developmental toxicity studies. These studies of clarified slurry oil demonstrate that this substance has much greater potential to cause developmental toxicity and systemic toxicity in repeat-dose toxicity studies than to cause effects on male and/or female fertility and reproductive organs.

In a developmental toxicity study of Sample F-179 (Table 5), groups of 11–18 mated female rats were given daily dermal doses of 0, 0.05, 10, and 250 mg/kg$_{bw}$/day starting at 7 days prior to mating through GD 20. The dams were allowed to deliver their litters, and the dams and litters were sacrificed on postnatal day 4. Maternal toxicity (decreased maternal body weight and absolute food consumption) was observed at 250 mg/kg$_{bw}$/day. The test material had a significant effect on the number of litters delivered at 250 mg/kg$_{bw}$/day; in fact, no pups were delivered at this dose. It appears that the test material caused all litters to be fully resorbed at 250 mg/kg$_{bw}$/day because the average number of implantation sites per dam was not significantly decreased compared to controls, indicating that these females had been pregnant and implanted normal numbers of embryos. The NOAEL for developmental toxicity in this study was 10 mg/kg$_{bw}$/day.

As described earlier, male and female reproductive toxicity screening studies of Sample F-179 produced no effects on male and female reproductive organs or fertility at a dose of 250 mg/kg$_{bw}$/day, a dose that was incompatible with fetal survival in the developmental toxicity study. Further, in a 90-day repeat-dose toxicity study of Sample F-179, no effect on male or female reproductive organ weights or histopathology was observed at the highest dose level of 530 mg/kg$_{bw}$/day. In comparison, the NOAEL for systemic toxicity in this study was reported to be 1 mg/kg$_{bw}$/day; effects observed at the LOAEL of 11 mg/kg$_{bw}$/day included thymic atrophy, increased relative liver weight, and decreased platelet count. Dermal application of 55 mg/kg$_{bw}$/day or greater produced clear systemic effects, including decreased red blood cell parameters, decreased platelet count, cellular depletion in the bone marrow, increased liver weights, hepatic congestion, necrosis and vacuolar changes in the liver, decreased thymus weights, thymic atrophy, chronic inflammation of the thyroid glands, and increases in blood urea nitrogen (BUN), cholesterol, creatinine, serum glutamic oxalic transaminase (SGOT) and alkaline phosphatase. Thus, the results of these three types of studies demonstrate that, for Sample F-179, male and female reproductive toxicity is not a sensitive parameter by comparison to systemic and developmental toxicity.

The difference between the results of the female reproductive toxicity screening and developmental toxicity studies of Sample F-179 is readily explained by the difference in the period of dosing. In the developmental toxicity studies, female rats were given the test substance during pregnancy, and effects on litter size were readily apparent. In comparison, the rats in the female reproductive toxicity screening study were given the test material up until

**Table 5**
Summary of developmental toxicity LOAELs and key effects observed at the LOAEL in developmental toxicity studies (Type II)[a] of HBPS administered dermally to rats.

| HBPS category | CAS no. | Sample no. | Dosing days[b] | Dev. Tox. LOAEL, mg/kg$_{bw}$/day | Percent pregnant | Gestation length | Implantation sites | No. of live pups per litter (PND 0) | Percent Survival to PND 4 | Pup body weight (PND 0) |
|---|---|---|---|---|---|---|---|---|---|---|
| Heavy fuel oil | 64741-62-4 | F-179 | PMD 7-GD 20 | 250 | – | – | – | ↓ | ND | ND |
| Heavy fuel oil | 64741-62-4 | F-229 | GD 0–20 | 50 | – | ↑ | – | ↓ | – | ↓ |
| Heavy fuel oil | 64741-57-7 | F-196 | PMD 7-GD 20 | 250 | – | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 64741-57-7 | F-197 | PMD 7-GD 20 | 241 | – | – | – | ↓ | ↓ | ↓ |
| Heavy fuel oil | 64741-57-7 | F-201 | PMD 7-GD 20 | 250 | - | - | ↓ | ↓ | – | ↓ |
| Heavy fuel oil | 64741-57-7 | F-225 | GD 0–20 | 150 | – | – | – | – | – | ↓ |
| Heavy fuel oil | 68783-08-4 | F-275 | GD 0–20 | 250 | – | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 64741-81-7 | F-200 | PMD 7-GD 20 | 50 | – | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 68410-00-4 | F-194 | GD 0–20 | 125 | – | – | – | – | – | ↓ |
| Heavy fuel oil | 68410-00-4 | F-215 | GD 0–20 | 500 | – | – | – | – | ↓ | ↓ |
| Undefined | Blend[c] | F-221 | GD 0–20 | 333 | – | ↑ | – | ↓ | – | – |
| Heavy fuel oil | 64741-61-3 | F-222 | GD 0–20 | 50 | – | ↑ | – | ↓ | – | ↓ |
| Heavy fuel oil | 64742-86-5 | F-227 | GD 0–20 | 333 | – | – | – | ↓ | – | ↓ |
| Heavy fuel oil | 64741-45-3 | F-228 | GD 0–20 | 1000 | – | ↑ | – | – | – | ↓ |
| Heavy fuel oil | 64741-81-7 | F-274 | GD 0–20 | 50 | – | – | – | ↓ | – | – |
| Heavy fuel oil | 64741-57-7 | F-276 | PMD 7-GD 20 | 250 | – | – | ↓ | ↓ | – | ↓ |
| Gas oil | 64741-82-8 | 87213 | GD 0–19 | >60 | – | – | – | – | – | – |
| Gas oil | 68915-49-7 | 86270 | GD 0–19 | 500 | – | – | – | ↓ | – | – |
| Gas oil | 68915-97-9 | 86271 | GD 0–19 | 125 | – | – | – | ↓ | – | ↓ |
| Gas oil | 64741-43-1 | F-193 | PMD 7-GD 20 | 259 | – | – | – | - | – | ↓ |
| Gas oil | 68334-30-5 | F-195 | GD 0–20 | 250 | – | – | – | - | – | ↓ |
| Gas oil | 64741-82-8 | F-199 | PMD 7-GD 20 | 1 | – | – | – | ↓ | – | – |
| Gas oil | 64741-59-9 | F-213 | GD 0–20 | 333 | – | ↑ | – | ↓ | ↓ | ↓ |
| Gas oil | 64741-86-2 | F-233 | GD 0–20 | >500 | – | – | – | – | – | – |
| Gas oil | 64741-82-8 | F-277 | PMD 7-GD 20 | 250 | – | – | – | – | – | ↓ |
| Crude oil | 8002-05-9 | 89645 | GD 0–19 | 2000 | – | – | – | ↓ | – | ↓ |
| Crude oil | 8002-05-9 | 89646 | GD 0–19 | 500 | – | ↑ | – | ↓ | ↓ | – |
| Aromatic extract | 64742-10-5 | 87476 | GD 0–19 | >2000 | – | – | – | – | – | – |
| Aromatic extract | 64742-04-7 | 86187 | GD 0–19 | >125 | – | – | – | – | – | ND |
| Undefined | Blend[d] | F-220 | GD 0–20 | >250 | - | – | – | – | – | – |

↓ = Statistically significant decrease; ↑ = statistically significant increase; – = no significant effect; ND = no data.

[a] Type II developmental toxicity studies are defined as those in which pregnant rats were exposed during gestation, litters were allowed to be delivered naturally, and the offspring were observed from the day of birth through postnatal day 4 (PND 4) or in two studies through PND 28.

[b] PMD = premating day; GD = gestation day.

[c] F-221 is a blend of seven HBPS(CASRNs 64741-45-3, 64741-57-7, 64741-58-8, 64742-67-2, 64742-78-5, 64742-86-5, and 68410-00-4).

[d] F-220 is a blend of three HBPS (CASRNs 64741-77-1, 64741-76-0, and 64741-75-9).

**Table 6**
Summary of the six type II developmental toxicity studies with a statistically significant decrease in the number of implantations per dam.

| HBPS category | Sample no. | Dosing period[a] | Dose,[b] mg/kg$_{bw}$/day | % Decrease in number of implantations (%) | Dev. Tox. LOAEL,[c] mg/kg$_{bw}$/day |
|---|---|---|---|---|---|
| Heavy fuel oil | F-200 | PMD 7-GD 20 | 250 | −25 | 50 |
| Heavy fuel oil | F-201 | PMD 7-GD 20 | 250 | −14 | 250 |
| Heavy fuel oil | F-222 | GD 0–20 | 500 | −18 | 50 |
| Heavy fuel oil | F-274 | GD 0–20 | 250 | −27 | 50 |
| Heavy fuel oil | F-276 | PMD 7-GD 20 | 250 | −6 | 250 |
| | | PMD 7-GD 20 | 500 | −24 | |
| Gas oil | F-199 | PMD 7-GD 8–11 | 250 | −14 | 1 |

[a] PMD = premating day; GD = gestation day.
[b] Dose level at which a statistically significant decrease in the number of implantations was observed.
[c] Lowest observed adverse effect level (LOAEL) for the most sensitive endpoint of developmental toxicity.

**Table 7**
Summary of the seven type II developmental studies of HBPS with a statistically significant increase in the average length of gestation.

| HBPS category | CAS no. | Sample no. | Dose, mg/kg$_{bw}$/day | Dosing period[b] | Increase in gestation length, days | Dev. Tox. LOAEL, mg/kg$_{bw}$/day |
|---|---|---|---|---|---|---|
| Undefined | Blend[a] | F-221 | 333 | GD 0–20 | 0.8 | 333 |
| Heavy fuel oil | 64741-61-3 | F-222 | 50 | GD 0–20 | 0.7 | 50 |
| Heavy fuel oil | 64741-57-7 | F-225 | 500 | GD 0–20 | 0.8 | 150 |
| Heavy fuel oil | 64741-45-3 | F-228 | 1000 | GD 0–20 | 0.7 | 1000 |
| Heavy fuel oil | 64741-62-4 | F-229 | 50 | GD 0–20 | 0.7 | 50 |
| Gas oil | 64741-59-9 | F-213 | 333 | GD 0–20 | 0.5 | 333 |
| | | | 1000 | GD 0–4, −5, or −6 | 0.7 | |
| Crude oil | 8002-05-9 | 89646 | 500 | GD 0–19 | 0.4 | 500 |

[a] F-221 is a blend of seven HBPS (CASRNs 64741-45-3, 64741-57-7, 64741-58-8, 64742-67-2, 64742-78-5, 64742-86-5, and 68410-00-4).
[b] GD = gestation day.

day 0 of gestation, and a Caesarean section was performed on GD14; no effects on resorptions, litter size or both were observed in this study. Thus, for this sample, it appears that dosing during gestation is necessary to cause an increase in resorptions and therefore a decrease in litter size.

Another sample of clarified slurry oil (Sample 86001) produced systemic toxicity at ⩾8 mg/kg$_{bw}$/day in a 90-day repeat-dose toxicity study of rats administered the substance dermally. Groups of 10 male and female rats were administered Sample 86001 at 0, 8, 30, 125, 500, or 2000 mg/kg$_{bw}$/day for 5 days/week for 13 weeks. The authors reported that the primary target organs of toxicity were the liver, thymus and bone marrow; the effects on these organs contributed to death, reduced body weights, anemia, and changes in the levels of serum chemicals and enzymes. The study authors identified a number of secondary effects of this test substance, including "possible hypoplasia of uterus, ovaries, prostate and seminal vesicles", as well as "focal hemorrhage in testes." A significant decrease in ovary weight was observed at 125 mg/kg$_{bw}$/day, a dose associated with a high rate of mortality in female (4/10) and male (7/10) rats. While there were signs of slight effects on reproductive organs in both male and female rats administered this dose, these effects were only observed at dose levels that produced a high rate of mortality. The lowest dose (8 mg/kg$_{bw}$/day) in this study was considered to be a LOAEL based on histopathological findings in the liver and thymus, while the LOAEL for reproductive organ effects in this study was 125 mg/kg$_{bw}$/day. In comparison, a Type I developmental toxicity study of the same sample (Sample 86001) reported a LOAEL of 8 mg/kg$_{bw}$/day for developmental toxicity (Table 4). Thus, for Sample 86001, effects on male and female reproductive organs were less sensitive indicators of toxicity than systemic and developmental toxicity.

### *3.4.2. Comparison of LOAELs for developmental toxicity and for reproductive organ toxicity in repeat-dose toxicity studies of the same samples*

Many (16) of the same samples of HBPS were evaluated in both developmental and repeat-dose toxicity studies. Table 8 compares the LOAELs for developmental toxicity with the LOAELs for reproductive organ toxicity in the repeat-dose toxicity studies. For two substances (Samples 120801, 60901), it was not possible to determine the more sensitive indicator of toxicity between developmental toxicity and reproductive organ toxicity since no effect in either type of study was observed at any dose. In comparison, it was possible to identify the more sensitive indicator of toxicity (i.e., lowest LOAEL) between developmental toxicity and reproductive organ toxicity for fourteen test samples studied in both types of studies. In the case of twelve samples, developmental effects were clearly a more sensitive indicator of toxicity than reproductive organ effects in the repeat-dose toxicity studies. In comparison, in only two studies (Samples 86181 and 8281) did effects on reproductive organs appear to be a more sensitive indicator than developmental effects. However, in both of these cases, systemic toxicity was clearly present at doses associated with effects on reproductive organs. In no case did an adverse reproductive tract finding occur at a dose lower than that producing developmental toxicity or other adverse effects in repeat-dose toxicity studies.

In the first study (Sample 86181), a 15% decrease in absolute epididymal weight was observed at 30 and 125 mg/kg$_{bw}$/day; a 13% decrease in cauda epididymis weight and a 16% decrease in sperm concentration per cauda epididymis relative to controls was seen at 125 mg/kg$_{bw}$/day, a dose associated with significant systemic toxicity.[1]

Systemic effects at 30 mg/kg$_{bw}$/day included decreased hemoglobin concentration (−6%), decreased hematocrit (−5%) and at week 13 increased relative liver weight (16%) and decreased thymus weight (−28%).

In the second study (Sample 8281), a 17% decrease in absolute testes weight was seen at 500 mg/kg$_{bw}$/day, and the prostate was described at necropsy as "small" in 8 of 10 animals at this level compared to 1 of 10 control males. However, evidence of systemic toxicity at this dose was pronounced, including a 29% reduction in terminal body weight and a 60% decrease in thymus weight relative to controls in males. Clinical signs of systemic effects at this

[1] Cauda epididymis weight and sperm/cauda epididymis were not assessed at 30 mg/kg$_{bw}$/day.

**Table 8**
Comparison of LOAELs for reproductive organ effects in repeat-dose toxicity studies and for developmental toxicity in studies of identical samples of HBPS.

| HBPS category | Sample no. | Malereproductive organs LOAEL, mg/kg$_{bw}$/day | Female reproductive organs LOAEL, mg/kg$_{bw}$/day | Semen evaluation LOAEL, mg/kg$_{bw}$/day | Developmental toxicity LOAEL, mg/kg$_{bw}$/day | | Develop-mental toxicity more sensitive endpoint? |
|---|---|---|---|---|---|---|---|
| | | | | | Type I | Type II | |
| Heavy fuel oil | 86484 | >30[a] | >30[a] | 500 | 8 | NS | Yes |
| Heavy fuel oil | 86001 | >125 | 125 (30)[c] | ND | 30 | NS | Yes |
| Heavy fuel oil | F-115 | >50 | 50(10)[c] | ND | 1 | NS | Yes |
| Heavy fuel oil | F-179 | >530 | >530 | ND | >0.05 | 250 | Yes |
| Heavy fuel oil | 83366 | >125[a] | >125[a] | >125[b] | 125 | NS | Yes |
| Heavy fuel oil | 86181 | 30(8)[c] | >125 | 125 | 125 | NS | No |
| Heavy fuel oil | 85244 | ND | >2000 | >2000[b] | 500 | NS | Yes |
| Heavy fuel oil | 10929 | 50 | 50 | ND | 25 | NS | Yes |
| Gas oil | 86271 | 500 (125)[c] | >500 | >500 | 125 | 125 | Yes |
| Gas oil | 86270 | >500 | >500 | >500 | 500 | 500 | Yes |
| Gas oil | 8281 | 500 | >500 | ND | >1000 | NS | No |
| Gas oil | 120801 | >600 | >600 | ND | >600 | NS | –[a] |
| Crude oil | 89646 | >500 | >500 | >500 | 500 | 500 | Yes |
| Aromatic extract | 86187 | >125 | >125 | >125[b] | 125 | >125 | Yes |
| Aromatic extract | 20906 | >150 | >150 | ND | 25 | NS | Yes |
| Lubricating oil basestock | 60901 | >1000 | >1000 | ND | >1000 | NS | –[a] |

ND = no data; NS = no study.
[a] Cannot be determined.
[b] Only sperm morphology evaluated.
[c] LOAEL(NOAEL).

dose included thin appearance and perineal staining. Hematological findings included a shift from lymphocytes to neutrophils, reduced hematocrit and hemoglobin concentration. Serum chemistry effects included increases in blood urea nitrogen and alkaline phosphatase and a decrease in lactate dehydrogenase.

## 4. Discussion

HBPS exhibited a low potential to produce male or female reproductive toxicity. There was little evidence of male or female reproductive toxicity in a large number of rat dermal repeat-dose toxicity studies, which evaluated the weights and histopathology of male and female reproductive organs, as well as semen quality in males. Similarly, a small number of well-conducted one-generation reproductive toxicity studies also did not identify male or female reproductive toxicity as a sensitive endpoint of toxicity for HBPS. In contrast, many samples of HBPS were shown to cause developmental toxicity when administered dermally to pregnant rats at relatively low doses, and to also produce systemic toxicity in repeat-dose dermal toxicity studies.

The studies presented herein provide substantial support for the hypothesis that effects in rats in dermal developmental and/or repeat-dose toxicity studies of HBPS occur at doses lower than those that might affect fertility in dermal one-generation reproductive studies. For most of the tested materials, no effects occurred on the reproductive system. In the few cases where some evidence of toxicity to reproductive organs was noted in repeat-dose toxicity studies, it was always associated with doses that produced other, often substantial, systemic effects.

A developmental toxicity study and a repeat-dose toxicity study appear to be adequate to predict the most sensitive effects that would be observed if a single-generation reproductive toxicity study was conducted on a HBPS. The sensitive effects on fetal development associated with HBPS would be readily identified in studies conducted according to testing guidelines such as an OECD 414 prenatal developmental toxicity study or an OECD 421 reproductive/developmental toxicity screening test. For example, in a large number of rat dermal developmental toxicity studies reviewed herein, many HBPS produced significant developmental effects, including decreased litter size and decreased fetal/pup body weight. A reproductive toxicity study, in which female rats are exposed during pregnancy, would be expected to demonstrate a similar decrease in litter size and pup body weight at birth. Such a study would not be necessary if a developmental toxicity study had already been conducted. Therefore, reliance on the results of developmental and repeat-dose toxicity studies presents a viable alternative to conducting reproductive toxicity studies in animals for HBPS. Dent (2007) evaluated the strengths and limitations of using repeat-dose toxicity studies of a wide variety of substances to predict effects on fertility. Among the 73 chemicals evaluated, repeat-dose toxicity studies accurately predicted the observed effects on fertility for 68 of the 73 chemicals.

In most of the repeat-dose toxicity studies evaluated herein, sections of selected tissues, including reproductive organs, were collected and preserved with 10% neutral buffered formalin, embedded in paraffin wax and subsequently stained with hematoxylin and eosin. At the time that these studies were conducted, formalin fixation of the testes was routinely employed by toxicology laboratories (Creasy, 1997). Subsequent to the conduct of these repeat-dose toxicity studies, regulatory agencies began to recommend that the testes be preserved in Bouin's fluid or modified Davidson's fixative rather than formalin to avoid shrinkage artifacts associated with formalin fixation and paraffin wax embedding that could interfere with the detection of subtle changes in the cellular structure of the testes (Aman, 1982; Chapin et al., 1984; Russell et al., 1990; Creasy, 2003; Lanning et al., 2002; Latendresse et al., 2002). It should be noted that histopathologists now look for more subtle effects than they did when most of these studies were performed. Subtle effects (e.g., sperm retention at basal lamina, positioning of developing spermatids with respect to the Sertoli cell, abnormal nuclear morphology, asynchrony of spermatogenic stages, Sertoli cell vacuolization) may be missed unless the observer has a thorough understanding of spermatogenesis. However, the chances of there being subtle effects after 90 days of treatment are thought to be low, especially if the weights of the testes and epididymis are unchanged from control weights (Lanning et al., 2002). Of note, five of the 90-day studies of HBPS, which were conducted more recently, used more current methodology for fixing and examining the testes, and testicular effects were not observed.

Repeat-dose toxicity studies of only 28 days duration were included in this evaluation since they were considered of sufficient

duration to discern effects on reproductive organs. This judgement was based on the results of a series of studies conducted by the Japanese Institute of Health Sciences and a large number of pharmaceutical companies to determine the optimal period and parameters for detection of male and female fertility disorders in rats. In males, it was concluded that a four-week daily treatment period is appropriate for detecting effects on male fertility, and that histological examination of the testes is the most sensitive approach (Takayama et al., 1995); in most cases, effects on male reproductive organs could be detected with a two-week daily treatment period (Sakai et al., 2000). In females, these investigators concluded that a two-week daily dosing period appeared to be sufficient to detect ovarian toxicity based on histopathological examination, except for cytotoxic compounds such as alkylating agents (Sanbuissho et al., 2009). Ulbrich and Palmer (1995) concluded from the results of a literature survey on the detection of effects on male reproduction involving 117 substances that prolonged treatment appears unnecessary because assessments using organ weight and histopathology conducted after 4 weeks appear no better than those conducted at or before 4 weeks. Thus, both the 28-day and 90-day repeat-dose toxicity studies were included in the current evaluation of reproductive organ toxicity to increase the power of the analysis.

Practically all of the one-generation reproductive, developmental, and repeat-dose toxicity studies of HBPS have been conducted in one species (the rat) using one route of exposure (dermal). The choice of the dermal route of exposure is considered appropriate since humans are most likely to be exposed dermally to these substances and since these compounds are absorbed through the skin and produce significant systemic toxicity in dermal toxicity studies in laboratory animals.

Although the rat is a common species selected for these types of studies, it is not known whether the rat is the most sensitive species for evaluating the reproductive toxicity of HBPS. Based on studies of intraperitoneal injections of individual PAHs, the mouse was more sensitive than the rat to the ovotoxicity of certain PAHs (Borman et al., 2000; Mattison, 1979). Thus, it is difficult to rule out the possibility that there could be species differences in HBPS toxicity.

Effects similar to those observed in the dermal developmental toxicity studies of HBPS in rats (i.e., decreased survival and growth) were observed in studies where HBPS were given to other species by the oral route of exposure. For example, in an oral developmental toxicity study of Prudhoe Bay crude oil in mice, decreases in live fetuses per litter and fetal body weight and an increase in resorptions were observed when pregnant mice were given 5 ml/kg of the test substance by gavage on single days of gestation (Khan et al., 1987).

In mink, a decrease in live kits per litter was reported in a single-sex, two-generation reproductive toxicity study in which female mink were fed diets containing 500 ppm (~65 mg/$kg_{bw}$/day) of either Alaskan North Slope crude oil or bunker C fuel oil for 60 days prior to mating through weaning (Mazet et al., 2001). These animals became pregnant at a normal rate, but were less successful in maintaining their pregnancies, had smaller litter sizes and decreased survival among their offspring. According to the study authors, the female offspring of the mink fed bunker C had significantly reduced reproductive success (i.e., decreased live-born kits per female bred) even though their only exposure to this petroleum product was *in utero* and during lactation; however, confidence in this finding is low due to the small group size (i.e., only 5 females bred) and lack of dose–response (i.e., only one dose level tested).

Male reproductive toxicity has been reported in rats given Nigerian crude oils orally; the results of these studies suggested histopathological alterations of the testes, decreased sperm count and reduced testicular weight in rats administered crude oil samples in drinking water or by gavage at doses up to 800 mg/kg/day Igwebuike et al., 2010; Obidike et al., 2007; Orisakwe et al., 2004). The oral route of exposure was chosen for these studies because oil spills have occurred on lands used for agricultural purposes in Nigeria and because crude oil is used by certain local populations in Nigeria as a folk medicine to treat a wide variety of ailments (Arikpo et al., 2010a,b; Orisakwe et al., 2004). A critical review of these studies is difficult to perform because the methods were not described in detail; some important outcome data including information on the health of the treated animals were not provided; also, there are inconsistencies between the tabulated information and the text of the studies.

In contrast to the studies of Nigerian crude oils, little evidence of male reproductive toxicity was observed in two 90-day dermal repeat-dose studies in rats of two samples of US crude oils (Feuston et al., 1997b). No changes in weight or histopathological effects were found in the reproductive organs even at the highest dose tested of 500 mg/$kg_{bw}$/day (Table 1). Also, epididymal spermatozoa morphology and count and testicular spermatid counts were unaffected by exposure to either of these crude oils at 500 mg/$kg_{bw}$/day (Table 2).

Different routes of exposure (i.e., oral vs. dermal) offer a possible explanation for the difference in the results of the dermal 90-day studies of crude oils compared to those of the oral studies of Nigerian crude oils. However, another HBPS (i.e., clarified slurry oil) was more toxic to mice when administered dermally than orally in a subchronic toxicity study (Feuston et al., 1997a). In another study, no significant increase in the incidence of abnormal sperm was found among mice injected intraperitoneally with Wilmington crude at levels up to 2.1 g/$kg_{bw}$/day for five consecutive days and sacrificed 35 days later (Lockard et al., 1982).

In one of the unpublished 90-day studies of aromatic extract (Sample 86187) evaluated herein, the test material was given dermally and orally (by gavage) to four and two groups of male rats, respectively. The study authors noted that the test substance appeared to be more toxic when given dermally than when given by gavage based on mortality, although at least one endpoint (i.e., prostate weight) appeared to be affected more by oral than dermal exposure. Prostate weight was not affected significantly at the highest non-lethal dermal dose (125 mg/$kg_{bw}$/day), but it was significantly decreased after oral exposure to $\geqslant$125 mg/$kg_{bw}$/day. Histopathological effects on the prostate were reported in the 500 mg/$kg_{bw}$/day orally dosed rats and in the 125 and 500 mg/$kg_{bw}$/day dermally dosed males; however, the orally dosed animals at 125 mg/$kg_{bw}$/day were not examined histologically. In addition, epididymis and seminal vesicle weights were significantly decreased in males given 500 mg/$kg_{bw}$/day orally, a dose which produced a significant decrease in body weight. Whether given orally or dermally, all doses that affected male secondary sex organs were associated with other systemic effects, including increased absolute and relative liver weights and decreased absolute and relative thymus weights, as well as effects on hematologic parameters and bone marrow histopathology.

The only available multi-generation reproduction study of HBPS was the study by Mazet et al. (2001) in mink, which had significant limitations, as described earlier. The study suggested an effect on fertility in the adult female offspring exposed *in utero* and during lactation to bunker C fuel oil, described above. There is evidence that exposure to at least one individual PAH (i.e., BaP) can suppress fertility among the adult offspring (both males and females) of pregnant mice exposed during gestation (Kristensen et al., 1995; MacKenzie and Angevine, 1981). Such an effect would not be discerned in a repeat-dose or developmental toxicity study or even a conventional single-generation reproductive toxicity study. It would require a multi-generation reproductive toxicity study or

an OECD 415 extended one-generation reproductive toxicity study to evaluate this possibility. However, scientists at the US EPA recently compared the parental, reproductive and offspring potencies from a large number of multi-generation reproduction studies to potency values for systemic toxicity from 90-day subchronic and chronic studies in the rat (Martin et al., 2009). The authors stated, "For the majority of chemicals, potency values between multi-generation, subchronic and chronic studies were comparable, with a general linear relationship falling within 10-fold of each other." Based on these results, it appears that a study to evaluate potential "second generation" effects on fertility of HBPS should be a low priority.

Pre-implantation loss was significantly increased in 6 of 30 Type II developmental toxicity studies of HBPS reviewed herein, although pre-implantation loss was not the most sensitive endpoint of toxicity in these developmental toxicity studies. Disruption of the processes that contribute to a reduction in fertilization rate and increased early embryo loss are usually identified simply as pre-implantation loss. The mechanism of the increase in pre-implantation loss in these studies is not known. The decrease in implantation sites was observed in studies that included exposure on GD 0 to 20, consistent with the experimental design used today for screening for developmental toxicity. Consequently, there would be no need to conduct reproductive toxicity studies to detect an increase in pre-implantation loss, since this effect can be readily detected in developmental toxicity studies when the dosing begins on GD 0.

Delayed parturition was noted in 7 of 30 Type II developmental toxicity studies of HBPS. In a review of inter-laboratory control data for reproductive endpoints, delayed parturition was associated with several factors, including dystocia, litter size and pup body weight (Marty et al., 2009). Among the Type II developmental toxicity studies of HBPS reviewed herein, delayed parturition was never observed in the absence of decreased litter size or reduced pup body weight at birth, or in most cases both. This suggests these factors played a role in the delayed parturition observed in the developmental toxicity studies of HBPS. In general, parturition in rodents is triggered by endocrine changes that are not shared with humans, making extrapolation of effects on this endpoint from rats to humans difficult and uncertain (Mitchell and Taggart, 2009). Arcaro et al. (2001) hypothesized that the delayed parturition observed in a developmental toxicity study of a sample of crude oil may have been due to anti-estrogenic activity of the test substance or to the induction or inhibition of estrogen metabolism. Regardless of the mechanism of action, it is important to note that delayed parturition in the rat was not one of the sensitive endpoints of toxicity in these studies; other effects were observed at lower doses in these studies.

Eventually, it may be possible to eliminate the need to conduct a reproductive toxicity study, even when developmental and repeat-dose toxicity studies of HBPS are not available, by modeling the results of such studies. Mathematical models have been developed for HBPS to predict the results of selected endpoints of 90-day repeat-dose and developmental toxicity studies. The use of the mathematical models represents a plausible alternative to animal testing. The strengths and weaknesses of using these mathematical models have been discussed previously (Murray et al., 2013; Nicolich et al., 2013; Roth et al., 2013; Simpson et al., 2013). When appropriate, utilizing computational modeling of repeat dose and developmental toxicity tests serves the goal of reducing the use of animals in testing. Obviously, the utility of this approach depends on the accuracy of the models to predict the repeat-dose and developmental toxicity of untested substances.

In conclusion, HBPS have demonstrated low potential to cause male or female reproductive toxicity relative to developmental toxicity and systemic toxicity in repeat-dose toxicity studies. Among 14 samples of HBPS, each of which was tested in both repeat-dose toxicity and developmental toxicity studies, there were no studies in which an adverse reproductive tract finding occurred at a dose lower than that producing developmental toxicity or other adverse systemic effects in repeat-dose toxicity studies. The available studies provide substantial support for the hypothesis that effects in developmental and/or repeat-dose toxicity studies of HBPS would occur at doses lower than those that might affect fertility in rat one-generation reproductive studies. Thus, when adequate data on developmental and repeat-dose toxicity are available for HBPS, a reproductive toxicity study of HBPS appears unnecessary for the purpose of satisfying the requirements of EPA's HPV Chemical Challenge Program.

## Conflict of interest

Four of the coauthors (RNR, BJS, MJN, FJM) are paid consultants to the Petroleum HPV Testing Group. Three are former (RNR, BJS, MJN) or current (LGR) employees of companies that manufacture petroleum products. One co-author (TMG) is employed by the American Petroleum Institute.

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