U.S. ARMY PUBLIC HEALTH COMMAND

Technical Guide 330

The Rodent Sperm Analysis Method in Terrestrial Health Risk Assessment

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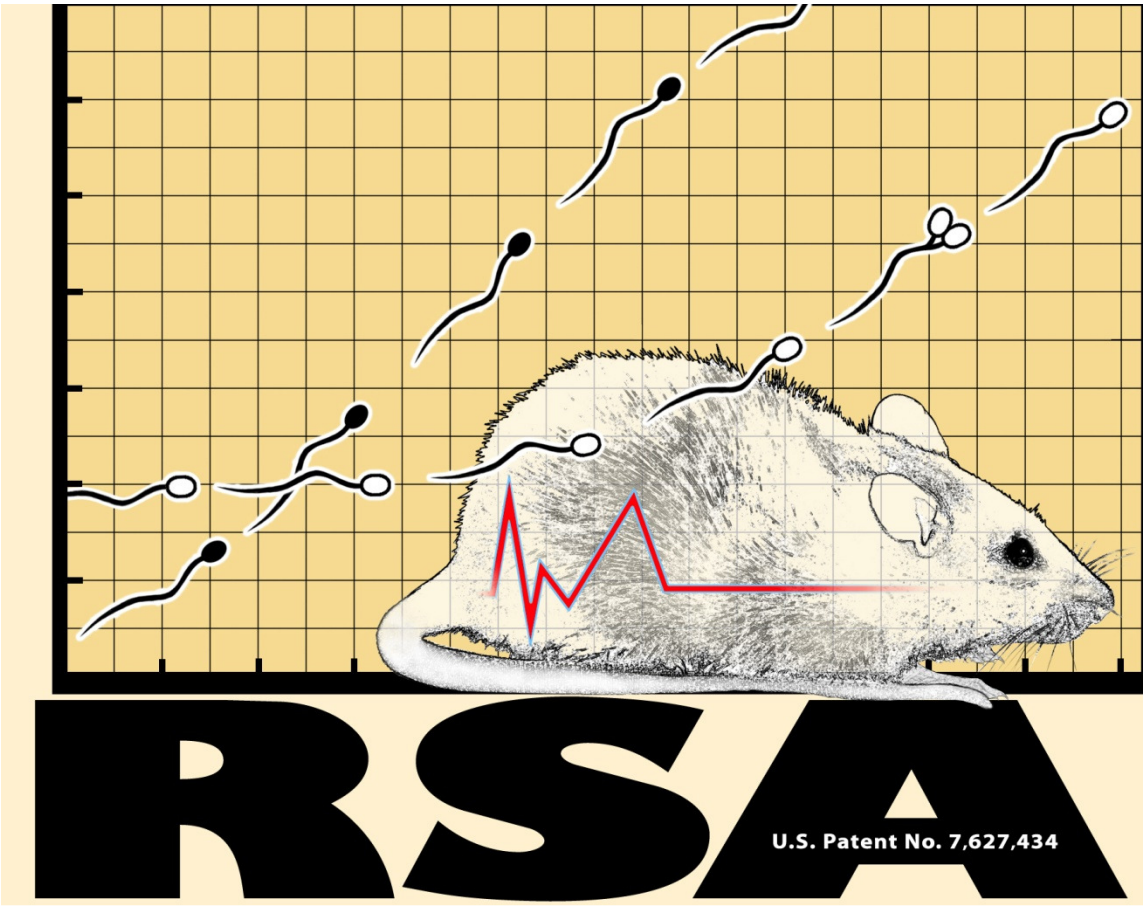

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Technical Guide 330
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PREFACE

The U.S. Army Public Health Command's Technical Guide 330 comprehensively presents the Army developed, U.S. Government patented Rodent Sperm Analysis (RSA) method in support of ecological assessments at contaminated terrestrial sites. This technical guide provides the theory of the RSA method, a description of the method's application, and cautious instruction with regard to the interpretation of RSA application outcomes conducted at Army installation.

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CHAPTER 1
OVERVIEW

The Rodent Sperm Analysis (RSA) method presented in this technical guide (TG) is a tool designed to assist conventional ecological risk assessment (ERA) approaches for mammals at contaminated terrestrial sites. It is critically important to understand at the outset, however, that RSA itself is not a risk assessment method. Thus, RSA does not forecast toxicological effects that could arise in site ecological receptors should sites persist in a nonremediated state where the receptors will otherwise experience continued chemical exposures. As is explained in Chapter 2, RSA is predicated upon an understanding that for the prototypical contaminated terrestrial site belonging to the Army, there is no need for anticipating or predicting health effects that might accrue to ecological receptors. Rather, there is a need to determine if ecological receptors at contaminated sites today are displaying health effects that have been elicited by the chemical exposures the receptors have experienced. It is equally important to understand that RSA applications are not toxicological, experimental, or research endeavors involving animals. Therefore, RSA applications do not involve: a) the purchase of laboratory-reared animals from suppliers; b) randomizing animals into treatment groups; c) assuming animal housing tasks such as adjusting diets, cleaning cages, and controlling indoor lighting and temperature; d) purchasing chemicals from suppliers; or e) administering chemicals as part of a dosing regimen. In stark contrast to toxicological investigations that support conventional ERA, RSA applications harvest animals from their natural settings, record diagnostic information from the animals that relates to ERA's toxicological endpoint of greatest concern (i.e., reproduction), and compare the recorded measurements to scientifically established and technically defensible standards. To better understand, the RSA's evaluative process can be compared to the case of a doctor reviewing the blood work for a new patient who had recently scheduled a well visit. For a patient who presented with a fasting blood glucose level of 350 milligrams per deciliter (mg/dl), the doctor will necessarily conclude that the patient has a compromised blood sugar metabolism, and the patient is diabetic. The determination in that case follows from the availability of a well-established 'norm' for blood glucose levels in fasted individuals and the knowledge that considerable exceedances of the 'norm' describe a diabetic condition. The RSA method is similarly able to render its useful reproductive capability determinations for mammals at contaminated sites because of the availability of established sperm parameter-based thresholds-for-effect that are used in a comparative assessment scheme (see Chapters 3 and 4).

The RSA method is the only existing direct health status assessment method for ecological receptors residing at contaminated sites. RSA advances the science of the conventional ecological assessments that are otherwise relegated to the use of food-chain and other models for the anticipation of health effects. Such models are almost always reliant on and limited to the use of contaminant concentration data for a site's environmental media (e.g., soil). The models therefore are quite removed from the 'whole organism' level of organization (i.e., from the actual site receptor). RSA is necessarily an impact or effects assessment method, intending to identify a key health effect should it arise in the mammals that occupy the contaminated sites. RSA can serve as a useful adjunct to a conventional assessment by furnishing what ERAs refer to as a 'line of evidence' (U.S. Environmental Protection Agency (USEPA) 1998). Additionally, RSA outcomes provide the strongest of such lines of evidence because they report on animals that have had unlimited opportunities for their reproductive biology to have been compromised (see Chapter 2).

The novelty of the RSA method is demonstrated with its status of being a patented process (U.S. Patent No. 7,627,434). The method's essential novelty is its capability to assign rather definitive health assessment determinations for the actual site receptor. In contradistinction, the conventional ERA process, since its inception, has only shown a screening capability for potential effects (Tannenbaum et al. 2003a). Consequently, the conventional ERA process can only allow for statements to the effect that a site receptor *could* develop a particular toxicological endpoint. The RSA method is novel for two additional reasons. First, it is the only method where outcomes reflect all three routes of contaminant uptake (i.e., inhalation and dermal contact in addition to ingestion), whereas current conventional assessment schemes only crudely track the ingestion route. Second, RSA is the only method that provides an assessment for chemical mixtures in soil. Since its inception, ERAs are only capable of reviewing chemicals singly for their potential to pose health effects.

CHAPTER 2
THE THEORY OF RODENT SPERM ANALYSIS

The RSA method was developed with the express intention of advancing the science of ecological assessment beyond the present design that has remained essentially unchanged for nearly three decades (Tannenbaum et al. 2007). The theory of RSA begins with a fundamental underlying premise, namely that if health effects were ever to arise in chemically exposed ecological receptors, those health effects would be evident today. The premise is reasonable and technically defensible having met with peer review acceptance on multiple occasions (Tannenbaum et al. 2003a; 2007; 2008). The premise is based upon two realities: a) by the time all terrestrial Superfund-type sites stand to have an assessment conducted, 30 years or more will have elapsed since contamination release events occurred (Tannenbaum 2002; Tannenbaum et al. 2003a), and b) ecological receptors have vastly shorter life spans than humans. Therefore, the realities support that as of the present day tens of generations of ecological receptors have already lived out their lives at contaminated sites. In the case of small rodents, at least one species of which is routinely evaluated in virtually all terrestrial ERAs, it is likely that more than 100 generations will have lived at a site by the present day. Importantly, RSA methodology duly notes that for any terrestrial receptor, and particularly so in the small rodent grouping (that encompasses a great many species throughout the United States), the ongoing breeding that has occurred over several decades (with allowances, of course, for immigration and emigration) has allowed for the fullest expression of site-posed toxicological effects. An extension of the fundamental underlying premise is that if health effects of concern are not evident today, it is unreasonable to suggest that these effects might still crop up some years hence (Tannenbaum et al. 2008).

RSA recognizes that given the ages of contaminated sites, irrespective of the environmental program under which sites may fall, there is no true need for assessing "risk" – the likelihood that a health effect will arise in a population. Further and in contradistinction, what needs to be assessed is whether or not an effect has already been elicited. En route to developing a method to accomplish just that, two essential decisions needed to be made: the specific receptor to be assessed for each field application had to be identified, as did a highly utilitarian toxicological endpoint to track. With regard to the first decision, RSA theory recognizes the numerous constraints that exist vis-à-vis well-intended efforts to collect biota (principally mammals and birds) from the field for use in any assessment scheme, understanding that invariably, destructive means must be used in conducting health assessment work (i.e., animal sacrifice is unavoidable). By way of example, although there may be a very keen interest to

evaluate fox or mink at a specific contaminated site, it is unrealistic to think that trapping these species would ever come to fruition. Animal care and use committees would frown on the collections. In the unlikely case where permissions were nevertheless granted to collect these animals, it is clear that the animals could not be removed from their habitat for any length of time. In the rare case where the trapping would be allowed to proceed, only minimally invasive sampling (e.g., removing a small bit of fur or drawing off a small blood sample) might be granted. Such samples would not allow for a health assessment, since empirical information does not exist to support health status assessments based on chemical detections in animal tissues. (Chemical detections in tissue would only indicate that chemical exposure is occurring.) It is important to note other independent and overarching reasons for forms such as fox and mink constituting inappropriate verification-of-effect species choices. First, animal collection would be inordinately time-consuming and labor-intensive, recognizing that some 15-20 specimens would probably be needed from both the site of interest and from a reference (i.e., contaminant-free) location in order to support a meaningful determination of some sort. Additionally, species such as fox and mink are not found throughout all habitats in the U.S., and considering all that is entailed in developing a utilitarian direct health status assessment scheme (see Chapter 3), it would not be prudent to develop one focused about animals that have less than a ubiquitous distribution. Additionally, species such as fox and mink have relatively large home ranges, and in the overwhelming majority of cases, these will exceed the site size by tens of times or more (Tannenbaum 2005a; USEPA 1993). Consequently, it would again not be prudent to develop an assessment scheme for what are clearly spatially irrelevant receptors in terrestrial settings.

While the small rodent grouping may, by default, be the only one that can be destructively sampled to support a direct health status assessment method, the grouping offers numerous advantages. Small rodents (e.g., mice, rats, and voles) are for all intents and purposes maximally exposed terrestrial receptors. Also, they are necessarily year-round (i.e., nonmigratory) species, are in continuous contact with soil, and have miniscule home ranges (on the order of 1 acre) that effectively lock them to a given site (be it a contaminated one or a reference location). There should certainly be no objection to using small rodents as sentinels in the mammalian health assessments of RSA field-based applications, when they already serve as sentinels for other mammals as part and parcel of the conventional desktop ERA process. (It should be noted as well that small rodents are used nearly exclusively as surrogate species in support of human health risk assessments; HHRA.) It is also true that laboratory studies with mice or rats form the basis of nearly all mammalian toxicity reference values used in conventional desktop ERAs (Sample et al. 1996). RSA theory reasons then, that if small rodents can be used to support the conventional ERA process that

does not venture to the out-of-doors to even observe small rodent activity, small rodents and particularly the ones that reside in the wild at sites of interest, can certainly be used to support field-based mammalian receptor health assessments.

The previous paragraph provides the basis for small rodents being valid surrogate species for the larger, wider-ranging, and often trophically higher mammals at contaminated sites (i.e., those for whose benefit site cleanups might realistically proceed). It is critically important to understand that RSA was not developed for the express purpose of producing reproductive capability characterizations for small rodents, since cleanups rarely, if ever, proceed in order to afford protection to these species. Two central points distill from the above discussion. First, there will probably never be a time when mammals other than small rodents (e.g., fox or badger) routinely, if ever, submit to collection for direct health status assessment. Second, although direct health status assessment for mammals appears to be relegated to work only with small rodents, the information furnished by an RSA application can be wholly sufficient to support a rather definitive reproductive determination for larger mammals (Tannenbaum et al. 2007). Therefore, in the aftermath of an RSA application, it would be inappropriate to comment to the effect that mammalian reproductive assessments will be considered incomplete until testing is done with larger site mammals. It is imperative to understand that such assessments will never be in the offing, and that presently (i.e., using the conventional desktop ERA process) remedial decisions based solely on rodent work routinely proceed.

Extrapolation on the basis of site fidelity (i.e., site presence), from the small rodent condition to those mammals that could support remedial actions, constitutes a key component of RSA theory. As mentioned above, small rodents are essentially locked to the contaminated sites where they are found because of their biologically set miniscule home ranges. The larger mammals that are of interest though have decidedly less contact with site-affected soils than rodents (Tannenbaum 2005a). Whereas a given rodent could conceivably spend its entire lifetime within the boundaries of a contaminated site, a larger mammal might only contact the same site less than 10% of the time. Further, on those occasions when a larger mammal is found standing on contaminated soil, it may not be feeding or preening itself there. RSA therefore employs the following extrapolation. If RSA application outcomes indicate that site rodents are not reproductively compromised (Chapter 4), by extension, the larger mammals with their lesser degree of site (i.e., soil) contact are also not reproductively compromised.

That reproduction should be the toxicological endpoint of choice to track with a direct health status assessment methodology is a straightforward argument. RSA theory reasons that overall ecological receptor health can be defined exclusively through reproduction. If a site mammal population is reproducing normally today (something that an RSA application can identify), it is known *de facto* that newborns are consistently attaining sexual maturity, finding mates, and again producing viable young. In this regard, RSA theory is highly consistent with the current ERA paradigm (USEPA 1997). Under that paradigm, the intention is to only calculate reproduction-based hazard quotients (HQ), ratios of assumed contaminant exposure levels to supposed safe exposure levels. Further, it is only when a reproduction-based toxicity reference value is lacking that an HQ reflecting a different endpoint (e.g., growth or behavior) is developed and applied (Tannenbaum 2005b). Such an ERA approach is saying that it is wholly sufficient to consider the lone toxicological endpoint of reproduction when drawing ERA conclusions. Importantly, RSA theory well acknowledges that toxicological effects other than reproductive ones (e.g., organ weight shifts, enzyme over or underproduction, and modified neurological function) may have been triggered by the site condition. Such effects are of no consequence though if reproduction can be shown to be proceeding in unimpeded fashion. (Note: The role of all nonsperm parameter-related information collected during an RSA field effort is addressed in Chapter 5.)

One other critical RSA theory element concerns the anticipated outcomes of RSA applications. Although each RSA application outcome must be judged purely on the data retrieved from the field and with no allowances for bias, the best information available suggests that it is highly unlikely that instances of reproductive compromise will be discovered at contaminated sites. An abbreviated list of phenomena that stand to pre-empt reproductive impacts from occurring at contaminated terrestrial sites include: receptor adaptation to a contaminated site condition over time, reduction in chemical toxicity with time, receptors having insufficient spatial and temporal contact with soil and affiliated media to trigger effects, and site populations having appreciably greater genetic diversity than laboratory test animal populations. In light of the above, one should be wary of just how easily RSA outcome information can be misconstrued. To date, the lack of instances of sperm parameter thresholds having been exceeded could cause one to wrongly conclude that the RSA method is incapable of detecting reproductive compromise when such is present. There are two relevant points to consider. First, sentiments expressed to the effect that RSA is insufficiently sensitive for the task at hand might reflect a bias in the form of an unwillingness to accept that ecological receptors at contaminated sites (to include maximally exposed receptors such as small rodents) may suffer no ill effects, reproductive or otherwise. Second, as RSA continues to be developed, a remaining objective is to test the method at a few

exceedingly contaminated sites with the express intention of 'forcing a failure' (i.e., creating an opportunity to discover an instance where one or more of the method's essential sperm parameter thresholds-for-effect are, in fact, exceeded).

RSA theory recognizes that for all terrestrial sites, as varied as they may be, the essential question to be answered is always the same: Are site ecological receptors reproductively compromised? With this focus, specifics with regard to a site's pattern of contamination do not need to be known, although ordinarily by the time an RSA application occurs, thoroughly detailed information has long been assembled. It should be recalled that RSA applications conclude only with findings of whether or not reproductive compromise has been identified; there is no accompanying discussion of site chemicals of concern. Where a reproductive effect at a given contaminated site *has* been discovered, it is beyond the scope of RSA to assign attribution of the effect to particular site chemicals. It bears repeating that with the anticipation that reproductive effects will <u>not</u> be discovered, there will be little need, if any at all, to relate RSA findings to the chemicals in a site's soil.

CHAPTER 3
CONDUCTING RODENT SPERM ANALYSIS

The RSA method is highly adaptable to the broad spectrum of environmental settings that exist and the broad spectrum of environmental programs where ecological health is a concern. Additionally, RSA can be applied at any point along the continuum of a given site's environmental investigation work. Most will elect to apply it after the conventional HQ-based approach has occurred where not unexpectedly HQs will often be found to exceed values of 1.0 (Tannenbaum et al. 2003b; Tannenbaum 2005b).

The RSA method has three minimum requirements: a) sites of interest have *bona fide* contamination footprints, b) contaminated sites support small rodent populations, and c) habitat-comparable reference locations that can submit to small rodent trapping exist near contaminated sites of interest. The paragraphs below expand on the latter two requirements.

Rodent Population Requirements

- If a site should primarily or only support shrews (technically, belonging to the mammalian order Insectivora and not Rodentia), the RSA method cannot be applied. Trapping success with most shrews is poor due to their miniscule body weight and exceedingly high metabolism. Occupied traps are unlikely to have the treadle tripped, thereby reducing trapping success. Shrews caught in live traps are often found in the early morning hours (when traps are checked) to be bloodied, near death, or expired; this from having desperately tried to escape the trap in dire search of food.

- Prior to field deployment, it must be known at what times of the year small rodent activity abounds. For at least one RSA application, success was achieved only because highly region-specific ecological conditions that bear on rodent activity had been reviewed and considered (Pathology Associates 2004; Tannenbaum et al. 2008).

- If threatened and/or endangered small rodent species are the only rodents present at a site of interest, the RSA method for obvious reasons cannot be applied. If threatened and/or endangered small rodent species are present in addition to rodents that are not special status species, procedures to follow need to be arranged with the state environmental agency that will be granting the animal trapping permit(s).

Reference Location Requirements

- At a minimum, a viable reference location has the same small rodent species of choice as that of the contaminated site (the target species). Although it is acknowledged that there may be no such thing as a perfectly matched reference location (USEPA 1994), a truly suitable reference location is one that appears to offer the same habitat as does the site of concern. The vegetation should, as closely as possible, match that of the contaminated site in terms of species and the percentage of cover. The reference location should be located as close to the contaminated site as possible, but far enough away to preclude a given rodent (an individual) from appearing at both locations. The intent of reference location selection in RSA is to locate a site where as many natural environmental factors (e.g., weather, slope, soil type, species and assemblages) align with those of the site of interest. Ideally, the only difference between the sites is that the reference location is free of contamination. In this way, should reproductive effects be discovered at the site of interest, it is possible to ascribe these to the affected site's chemical-in-soil footprint. It is acknowledged that RSA outcomes reflect the totality of stressors present at a site of interest, which may extend to more than chemicals in soil (Tannenbaum et al. 2007).

- It is strongly recommended that at least a second ('backup') reference location be identified prior to deploying to the field to apply RSA, as is the recommendation in supporting guidance for field-based ERA efforts for Superfund work (USEPA 1994). Although trapping for an RSA application could logistically commence at the reference location, it is important to always remember that it is the rodent species (one or more) present at the contaminated site that must also be present at the reference location, and not the other way around. A potential reference location that by all appearances should have the target species residing there may not.

Other essential RSA method elements are discussed below.

Preliminary Site Visit

Procedurally, an RSA application begins with a preliminary site visit to allow those individuals who will be conducting RSA tasks in the field to gain a familiarity with the site environs. The preliminary site visit should accomplish the following tasks:

- ascertain the boundaries of the affected site;

- identify specific areas at which to place animal traps;

- identify two or more nearby suitable reference locations sufficiently distanced from the contaminated site to preclude the possibility of target species 'crossover' (i.e., the ability of a target species representatives to appear at both the contaminated site and the reference location);

- identify a practical and desirable location for deploying a mobile onsite laboratory;

- establish the probable small rodent species list for the areas to be animal-trapped;

- identify the hospital nearest the study site, and the quickest travel route from the study site to the hospital;

- locate a nearby vendor for a carbon dioxide tank and regulator gauge (materials needed for animal euthanization; see Sperm Motility Assessment below); and

- locate a certified facility at which animal waste (carcasses) and medical waste ("sharps") can be disposed of.

It is recommended that the preliminary site visit occur shortly before the actual RSA field work commences so that site familiarity (e.g., recognition of landmarks) is maintained. By way of example, an area in full leaf-out will look vastly different from the same area either before leaves have emerged or after leaves have dropped.

Animal Handling / Animal Care and Use

All animal handling, euthanization, organ harvesting, and tissue disposal procedures are addressed under U.S. Army Public Health Command's (USAPHC's) Animal Care and Use Committee Protocol #11 - 09 - 02 (USAPHC 2011). It should be noted that in addition to addressing RSA requirements, this protocol contains study elements for a female reproductive assessment scheme involving ovarian follicle counts.

Animal Trapping Permits

The appropriate state agency that issues animal trapping and animal take permits should be contacted early. All agency forms should be filled out and returned well before field deployment.

Animal Trapping

Although RSA is a male-based assessment method, considerable population-descriptive information from female rodent captures is also collected (see Population Data below). As explained in Chapter 5, this data can often serve in a corroborative capacity (i.e., to supplant the sperm parameter-based determination that will be made).

Although animal trapping at a contaminated site of interest and at a matched (noncontaminated) reference location can occur concurrently, as a rule, it is advisable to trap first at the contaminated site, since it is the rodent species occurring at the contaminated site whose reproductive health is to be assessed. Pre-selected reference locations therefore will only be demonstrated to have been viable ones for RSA purposes when it is noted that they have yielded the same species found at the contaminated site of interest. Live animal traps should be set out in a saturated scheme (i.e., many traps within a relatively small area), and to the extent practicable, with the same array used at both the site and reference locations. A recommended array for either a contaminated site or reference location has four 100 meter (m) x 100 m grids with each grid having 100 traps aligned in 10 parallel rows of 10 traps each (see Figure 1). Two of the four grids at a location (either the contaminated site or the reference location) should be trapped for three consecutive nights (unless requisite capture numbers occur sooner than this), after which the remaining two grids are trapped for another three nights or until the requisite number of animals are obtained. Traps should be spaced 10 m from each other in both horizontal and vertical rows. Traps should be set (i.e., with spring-loaded doors in the down position) in the late afternoon and appropriately baited for the rodents of the locale where the field work is being conducted. A small cotton ball should be placed inside the trap to provide comfort to captured rodents. Traps should be checked for captures at daybreak to minimize thermal stress to animals (particularly for those rodents that may have been trapped shortly after traps were set). Individuals checking traps must wear gloves, and traps should be held downwind at arm's length for the initial assessment. Trapped animals at the point of capture are transferred to a plastic bag for identification to species, sex, and age (as juvenile, sub-adult, or adult). General health condition should also be recorded in a field notebook. All captures are weighed in the plastic bag using an appropriate scale. All captures other than adult males have a small patch of fur on the back removed with scissors (done in an effort to circumvent the problem of double-counting animals over what may be as much as 2-week period of trapping) and are then released in the field at the point of capture. Adult males to be assessed are transferred back to their traps and conveyed by open truck to the onsite laboratory after all grids have been checked for captures. Over the course of the field effort, the desirable goal is to capture at both the contaminated site and its matched reference location, 15 adult males (target

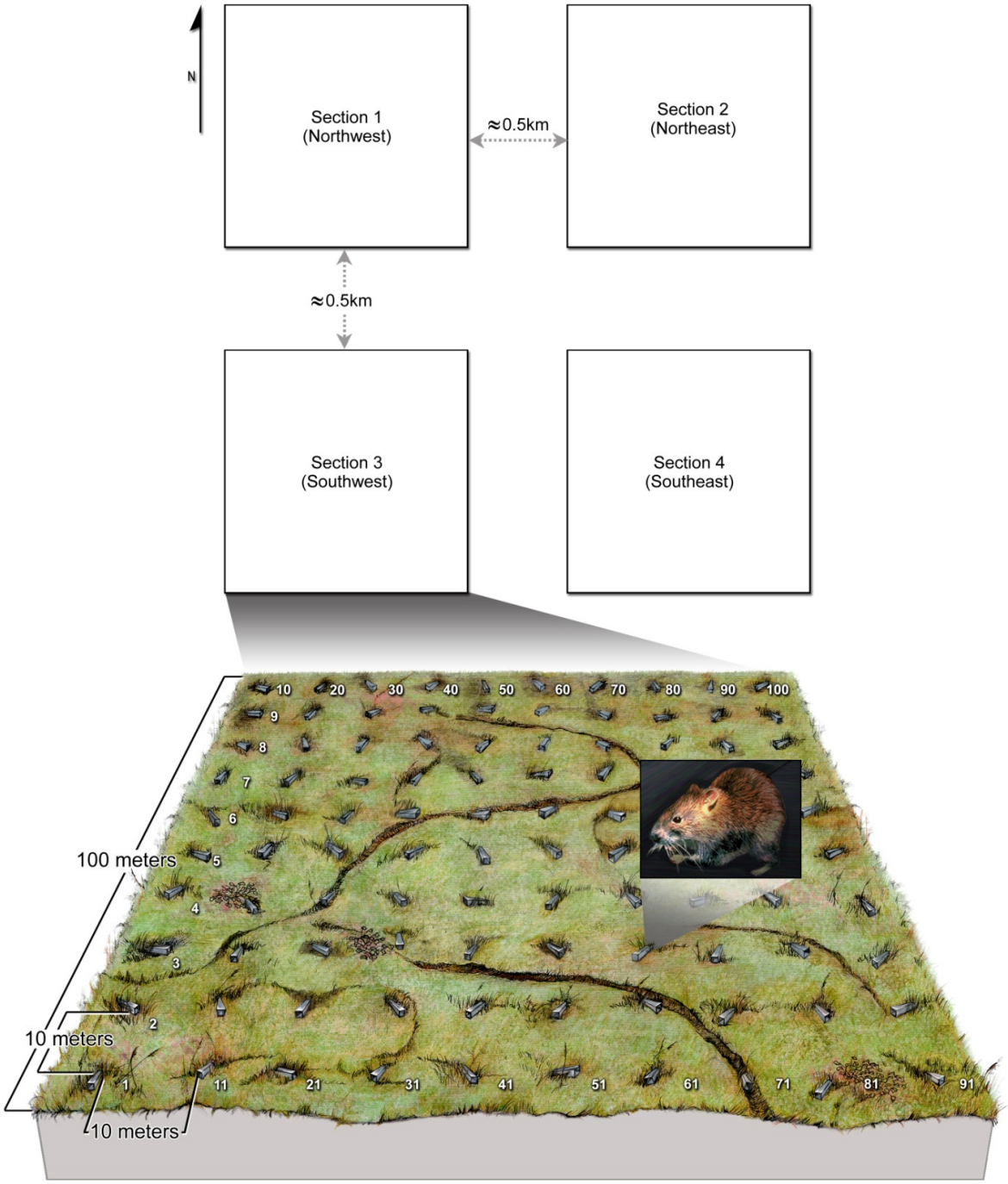

Figure 1. Sample Trapping Grid and Animal Trap Placement Schematic

animals) of at least one commonly occurring species. Note that a lesser capture number (e.g., 8 - 10) can supply information that is equally valuable to that of a 15-animal dataset. A smaller dataset may reflect that the entire male population for the site of interest, or a figure very close to this, has been sampled. Such can be confirmed when reviewing animal capture data over the latter trapping days of an RSA application. An observable reduction in the adult male capture rate and a concomitant increase in the number of recaptured animals (i.e., fur-clipped juveniles and/or females) over the latter trapping days may suggest that most of the adult males in the vicinity have been already culled (Tannenbaum et al. 2008).

Generally in RSA deployments, it will be observed that there is one dominant small rodent species present. It is possible though for more than one rodent species to be collected in sufficient numbers to allow for multiple species comparisons. However, if after the first several days of animal trapping there are only two or three adult males of what is emerging to be an “occasional” (as opposed to a dominant) species, all future captures of that species should be released each morning when traps are checked, and after sex, age, and body weight information has been recorded.

Data Collection

The data to be collected with an RSA application fall into three distinct categories. The primary category consists of three sperm parameter measurements of the adult male rodents that are captured in the field. The two secondary categories are population-level data (referring to a characterization of the field rodents in terms of species, sex ratio, and age distribution) and tissue-level data (principally comprised of internal organ weights). It is important to understand that the sperm parameter data alone drive the determinations of site mammal reproductive health or lack thereof. Strictly speaking, the other two data types do not need to be collected. Where the data are available, however, such should be provided to the customer along with a caveat as follows. Population-level and tissue-level data will likely corroborate the sperm parameter-based findings. Should this not be the case, the sperm parameter-based determination that is made is understood to be no less defensible.

It is critical to note that even with regard to the sperm parameter information that allows for essential RSA determinations, an adjustment to the scope of the data collection can be made. Specifically, a customer may opt to dispense with the assessment of sperm motility. Although generally a more inclusive dataset is to be preferred, there are reasons to find the sperm motility measure not to be the most vital to RSA assessments. First, historically the threshold-for-effect for motility has yet to be

reached, suggesting that motility is amply buffered so that even under highly contaminated site conditions notably reduced motilities are unlikely to be discovered. Second, it may be that computer assisted sperm analysis (CASA) equipment that can conveniently assess the motility will not be able to be procured for a given RSA job. Whereas sperm count and sperm morphology measures can be adequately assessed without the use of modern CASA equipment, CASA is rather indispensible for the collection of motility data. Cost considerations may also impact a decision to dispense with sperm motility, and thereby collecting only sperm count and sperm morphology information. The proper assessment of motility necessitates rather highly trained and experienced technicians to be present at the time animals are euthanized for as many days as target animals are being brought in from the field for sperm analysis, and labor costs for such technical support could present itself as an issue for the customer. It is equally important to recognize that a histological (histopathological) review is not absolutely essential for an RSA application. Dispensing with such an analysis can also help to make RSA affordable where budgetary constraints are an issue. It is useful to note that even where an RSA application includes an evaluation of all three sperm parameters (i.e., including specialized contractor support to collect and analyze sperm motility data) the cost of the effort is considerably less than that of a more conventional desktop ERA.

Sperm Parameter Assessment – General

The three sperm parameters generally evaluated in support of RSA's reproductive assessments are count, motility, and morphology. The motility measure is the only one that must be conducted at the time of euthanizing. Unlike count and morphology, motility has a very delicate assessment procedure in terms of being highly temperature-sensitive and requiring rapid and rather precise timing for the generation and processing of samples. For the count and morphology measures, tissue samples can be kept frozen and stored away for analysis at a later time. At a minimum, animal euthanization, harvesting organs, and the sperm motility determination occur inside a mobile laboratory, positioned at a convenient location on the Army installation. The mobile lab's fume hood and associated venting system ensure respiratory protection (e.g., from Hantavirus) to those working directly over euthanized animals. Sperm analysis occurring either in the mobile lab or at a later time at another location is ideally conducted with a Hamilton Thorne integrated visual optics system (IVOS) sperm analyzer.

Sperm Motility Assessment

Immediately following euthanization with carbon dioxide accomplished in a sealable enclosure (e.g., plastic bag, Styrofoam crate), a final animal weight is recorded, and the dissection begins. The right epididymis is immediately (i.e., within a minute and a half) excised with care to minimize blood contamination and is placed into a pre-warmed (36-37 degrees Centigrade (°C), but absolutely not higher) Petri dish containing 3 milliliters (mL) of a solution of 1% bovine serum albumen dissolved in phosphate buffered saline. The caudal portion is punctured twice with the tip of a #11 scalpel blade to release sperm, commencing a 3-minute "swim-out" period. After the swim-out, the dish is gently swirled, and a 9 microliter (µL) sample from a fairly dense portion of the sperm cloud is placed onto an appropriate slide (e.g., 20 u 2X-CEL slide), and the slide placed onto the pre-heated (37°C) stage of the IVOS. To facilitate measuring motility, the IVOS should be programmed to assess the sample at five fields along the length of the slide and to provide an average motility value for the five measurements.

Sperm Count and Sperm Morphology

Measurement of these two nonmotion parameters derive from a preparation of the left epididymis, and the two measurements are conducted in tandem. The freshly excised left epididymis can be worked up for analysis in the mobile lab after the motility measurement is completed, or alternatively, it can be wrapped in aluminum foil and kept frozen for workup at a later time. In either case, after precisely weighing the caudal portion of the room temperature epididymis (i.e., to four decimal place accuracy), the preparation begins with mincing the tissue a few times in a Petri dish containing 3 mL of deionized water. The dish is gently swirled to allow cells to be liberated. Two drops of the dish contents are removed to each of two standard glass microscope slides, and the edge of a clean glass slide is gently dragged across each of these to establish a thin layer of sperm cells which are first set aside to air dry. The slides are later stained in a 5% eosin bath and cover-slipped for microscopic evaluation. Two hundred sperm cells are evaluated with reverse phase/dark field microscopy for head and tail abnormalities (size, shape, and double head/tails), with the results reported as the percentage of abnormally shaped (sperm) cells in a population of 200 sperm. To circumvent bias in counting (i.e., having too many cells present in a single field of view), a 20x objective should be used.

Following slide preparation, the sperm count procedure proceeds as follows. The contents of the Petri dish (i.e., the caudal epididymis and the 3 mL solution minus the two drops removed as part of the morphology measurement) are then carefully and

completely transferred to an appropriate-sized soft plastic centrifuge tube. The Petri dish is rinsed with several additions of deionized water to maximize the fullest transfer of all sperm cells to the tube bringing the final tube volume to exactly 10 mL or 30 mL for mice and rats, respectively. A homogenizer probe is inserted into the tube and the contents are homogenized on a medium setting (approximately 20,000 rpm) for approximately 90 seconds. A 100 µL sample is withdrawn and added to a commercially available plastic 1.5 mL snap tube containing a fluorescent dye, where the dye has been activated several minutes prior with the addition of 100 µL of deionized water (for a total liquid volume of 200 µL in the snap tube). After 10 minutes is allowed to elapse (during which the dye stains the sperm heads), the closed snap tube (with its combined contents of 200 µL) is briefly vortexed. A 9 µL sample is removed by pipette to an appropriate microscope slide for the programmed IVOS to count sperm in five fields, and to instantaneously provide summary statistics. Of note, the error rate with the IVOS (i.e., the likelihood of counting debris in the slide preparation as sperm) for the system, as described, is significantly less than 5%.

Population Data

Applying the earlier described saturated animal trapping scheme will likely result in numerous animals being caught, many of which are not target animals. Field notebooks should record all nontarget animal information, to include total number of species caught, total capture numbers, sex, and age designation (as juvenile, sub-adult, adult). At a later time, sex ratio and overall population age structure information can be compiled. To facilitate the population characterization, albeit one that is based on a brief and singular trapping effort, all nontarget animals need to have a small patch of fur removed from the back so that there will be no double-counting in the capture data. Note that the phenomenon of greater or lesser capture success at a contaminated site relative to that of a reference location may be attributable to factors unrelated to a site's contamination (e.g., a recent precipitation event; trees of different species at the two trapping sites).

Tissue Data

After the two epididymides have been removed and processed, the following organs should be removed, blotted dry, weighed to four decimal places, and placed into small, individually marked glass vials of preservative: the liver, the spleen, the paired kidneys, the paired testes. (Note: In the event that a histopathological analysis is to be conducted at a later date, see Chapter 5.) [The left epididymis weight will have been

precisely recorded when it was first harvested for the purposes of assessing sperm count and sperm morphology (i.e., before the caudal portion was excised)]. For constructed organ-to-body weight ratios, if notable and statistically significant differences should be observed between the contaminated site and reference location, it will not be known if such differences signify a health effect. Information that would relate organ-to-body weight ratios to health effects does not exist. Potentially, site-exposed rodents with statistically significant oversized or undersized organs and organ-to-body weight ratios that are seemingly askew because of these differences could actually be healthier than their reference location counterparts.

CHAPTER 4
RODENT SPERM ANALYSIS COMPUTATIONS

This TG avails itself to the existence of established biologically significant thresholds-for-effect. For each of the three monitored sperm parameters, the degree of change in contaminated site rodents (relative to rodents of an appropriate reference location) that signifies reproductive impairment is known. As a consequence, there is no need to apply conventional statistical analyses (e.g., Student's t test, Analysis of Variance; ANOVA) in reviewing any observed degrees of sperm parameter change. Such analyses can be misleading and can contribute to erroneous findings when statistically significant differences are assumed to reflect biologically significant ones.

The three sperm parameter-based thresholds-for effect are all to be applied in a comparative scheme. At a minimum, a contaminated site rodent population's sperm count would need to be 80-90% less than that of a reference location to suggest that reproduction is being compromised (Chapin et al. 1997; Bucci and Meistrich 1987; Gray et al. 1992; Meistrich et al. 1994). In light of accounts that reproductive compromise can be triggered at the lesser rate of a 60% relative sperm count reduction, this latter figure is applied for conservatism in the assessment scheme (Tannenbaum et al. 2007). Importantly then, sperm count reductions less than 60% are inconsequential, and even if such should be highly statistically significant. In addition to this employed sperm count threshold-for-effect, a 40-50% motility reduction in site-exposed animals also signifies compromised reproductive success, as does a relative 4% increase in the frequency of misshapen sperm (i.e., sperm that are bent, broken, or two-headed; Chapin et al. 1997).

There are four computational elements to an RSA reproductive assessment for site mammals. For each of the three sperm parameters, the first step is to compute the arithmetic mean for the target animals of the site and reference locations. The second step is a simple comparison of the parameter means to identify instances of parameter shift in contaminated site rodents (relative to reference location rodents) that are not in the direction of favorability (i.e., a lesser sperm count, a lesser sperm motility, a greater incidence of morphologically altered sperm). If parameter mean differences in contaminated site rodents are all favorable, there is no need for further RSA analysis. Such an outcome can be considered constructive weight-of-evidence that site rodents, and by extension other site terrestrial mammals, are reproductively sound. If however, there should be one or more parameter mean differences that are not favorable for site rodents, the third step is to calculate the percentage increase or decrease in the parameter for the animals. The fourth step is to compare the noted shift (i.e., the percent increase or decrease) with the thresholds listed in the previous paragraph. If

just one sperm parameter threshold is exceeded, it is concluded that site rodents and all other site-influenced mammals are experiencing compromised reproductive success. This conclusion is drawn despite the reality that the larger and wider-ranging terrestrial site mammals (i.e., those species, unlike small rodents, for whose protection site cleanups *can* realistically proceed) will have a substantially lesser degree of direct site (ostensibly, soil) contact than the rodents.

Tables 1a and 1b illustrate how the necessary sperm parameter data should be arranged to facilitate the above computational steps. As the example illustrates, on occasion sample sizes may vary for a site's three-way sperm parameter analysis. Regarding the tabular data presented, it may be that a complication arose when using the right epididymis for the motility analysis, and a decision having subsequently been made to use the left epididymis to still secure the motility information. Such a situation would not allow for the count and morphology measurements to be gleaned. (Note: Remember that sperm count is generally the most valuable sperm parameter to track in RSA work, because it is commonly found to be somewhat suppressed in the contaminated site animals of most RSA applications. In contrast, sperm motilities have most often been found to be higher in contaminated site animals although not demonstrably so and without statistical significance.) Per Table 1a, the population means for sperm count and morphology in contaminated site animals indicate shifts that are not favorable (i.e., the count is lowered, and there are more misshapen cells). In contrast, the population means comparison for sperm motility in Table 1a indicates a shift for the contaminated site that *is* favorable. As a consequence, there is no need to assess sperm motility any further. For whatever the chemical stressors may be at the contaminated site, these stressors have not acted (singly or in combination) to impact or impair sperm motility. The information provided in Table 1b facilitates the evaluative sequence for sperm count and morphology following Table 1a's comparison of parameter means. The relative changes in the parameter means are computed, and these changes are compared with the parameters' respective thresholds-for-effect. The RSA data in Table 1b support a conclusion that the Hispid cotton rats of the contaminated site are not reproductively compromised. By extension, it is concluded that the larger site mammals that constitute a true concern for site stakeholders are also not reproductively compromised. In RSA's conservative approach to assessment, exceeded sperm parameter thresholds, even if not statistically significant, are interpreted to mean that reproduction is being compromised.

It is important to recognize that the threshold-for-effect for sperm motility, unlike that for sperm count or sperm morphology, is expressed as a range, specifically as a 40-50% relative decrease. Although unlikely (based on past RSA outcomes), it is possible that

Table 1a. Sperm parameter comparisons facilitating an RSA determination (Species: *Sigmodon hispidus*, Hispid cotton rat)

Sperm count (10^6sperm/gram of cauda epididymis)			Sperm motility (% motile cells)			Sperm morphology (% misshapen cells/200 ct.)	
Reference location	Contaminated site		Reference location	Contaminated site		Reference location	Contaminated site
2592.4	1991.0		83	70		0	3
1515.1	1859.9		57	73		1	0
2153.9	2139.0		83	94		0	2
1931.5	1674.7		80	83		2	3
1919.3	2958.4		69	59		1	2
1889.9	2152.6		74	93		2	3
2496.9	1513.8		85	85		3	1
2157.5	1628.2		51	86		3	0
2124.5	2181.6		16	95		0	2
2027.5	933.3		65	90		0	0
2019			81			0	
3264.7			30			2	
1994			77			1	
1638.5			81			7	
1757.5			68			0	
1975.7			68			1	
3471			77			1	
2094.7			61			0	
2054.8			70			2	
			53				
mean: 2162.0	mean: 1903.3		mean: 66	mean: 83		mean: 0.7	mean: 0.8
n=19	n=10		n=20	n=10		n=19	n=10

Table 1b. Evaluative sequence following sperm parameter population mean comparisons

Sperm parameter	Relative change in parameter in site animals	Threshold-for-effect	RSA Determination
Count	12% decrease	60% decrease	Threshold not exceeded
Morphology	0.14% increase	4% increase	Threshold not exceeded

observed relative motility reductions in site rodents could fall within this specific 10% range. Should such occur, and should the other two sperm parameters not reveal threshold exceedances, best professional judgment should be applied in concluding whether or not reproductive compromise is occurring in site mammals. It is important to recall that compromised reproduction, if it should exist at the site of interest, will ordinarily take the form of such things as less mating, fewer litters, and smaller-sized litters, as opposed to reproduction shutting down entirely. To the extent that rodents observed at a site are not exclusively (relatively) new immigrants, their presence (i.e., evident in having been captured in sufficient number to support an RSA application) may be providing a first indication that reproduction is proceeding normally or adequately.

On very rare occasion target animals from either a contaminated site or a reference location will be found to have no sperm at all, a condition known as azoospermia. Where such occurs, this information should be documented in an RSA application's Male Reproductive Assessment Report (MRAR; see Chapter 5). While the MRAR may report the percentage of a site's target animals that had this condition, it is important to recall that: a) it is not known what percentage of azoospermic males in a population poses a reproductive concern, and b) such a computed statistic would not factor into an RSA determination. Regarding standard RSA computations, where azoospermia is found to occur, values of 0 (reflecting 0 sperm counts) should not be averaged in with other positive sperm counts when expressing a population's average sperm count. This matter is discussed in Chapter 5. Tables 2a and 2b provide a review of a sperm count computation where azoospermia was actually encountered at a contaminated site.

Table 2a. Sperm count computation for a population with Azoospermia (Species: *Sigmodon hispidus*, Hispid cotton rat)

Sperm count (10^6 sperm/gram of cauda epididymis)	
Reference location	Contaminated site
184.9	0
466.8	0
494.6	184.5
563	2414.1
707	0
711.5	0
829.7	0
1008.6	953.1
1267.4	0
1284.9	0
	0
	2024.9
	0
	0
	0
mean: 951.3	mean: 1394.2
n=10	n=4 (azoospermic animals discounted)

Table 2b. Evaluative sequence following sperm count computation for a population with Azoospermia

Sperm parameter	Relative change in parameter in site animals	Threshold-for-effect	RSA Determination
Count	73.3% increase	60% decrease	Threshold not exceeded

CHAPTER 5
INTERPRETING RODENT SPERM ANALYSIS RESULTS

In reviewing and considering the information brought forward through ERA investigation, there is the potential for ecological risk assessors and risk managers to display bias. Bias that can interfere with due consideration being given the information furnished by RSA applications takes several forms. These include: believing that HQs express risk; believing that HQs >1.0 indicate harm-inducing environments; believing that HQs >1.0 sanction remedial actions; expecting and/or intending all contaminated sites to ultimately need a cleanup in order to provide adequate safety for ecological receptors; and unwillingness to accept that mammal-based RSA outcomes are as definitive as are possible given certain constraints. An overarching bias is the out-of-hand rejection that it is possible for ecological receptors to be chemically exposed and yet not bear any negative health effects. For various reasons, risk managers may not want to hear that contaminated sites are problem-free. Where biases such as those listed here are known to prevail, there is seemingly little point to conducting RSA, for RSA applications are likely to show that maximally exposed mammals are adequately protected at soil-contaminated sites.

If it is known *a priori* that site stakeholders will not value RSA results, justification for conducting RSA nevertheless exists. With each RSA application that is conducted, additional useful sperm parameter-based information is gained, and this is compiled into the USAPHC's proprietary database of RSA outcomes (Teresa4). It is anticipated that as the database grows, already established trends will become further elucidated and documented. Stakeholders and regulators who may be skeptical of RSA's merits may acknowledge the concepts of: a) decades-old contaminated sites "as is" being protective of their ecological site receptors, and b) RSA constituting a worthwhile ERA adjunct.

RSA results speak only to reproductive health of site mammals (and by extension to the total health of these receptors). Although mammals comprise only one of the two terrestrial receptor groups evaluated in ERAs (avians being the other), the utility of the method's results should not be underestimated (i.e., RSA results have far more information to offer than chemical-specific HQs). Additionally, in a generic way, RSA findings can be used to also draw inferences about avian health at a contaminated site (see Chapter 8).

The MRAR is the reporting format of choice for RSA applications, fostering the interpretation of RSA method results. The MRAR generally follows the format of papers published in peer-reviewed scientific journals. In the Introduction, the specifics of the

RSA application are briefly discussed. The specific Army installation that is the subject of the investigation and the specific portions of the installation that were studied are identified. The actual species that were collected in sufficient numbers to support the effort and the corresponding trapping dates are listed. The narrative should document any complications that arose, such as rain events that interfered with the intended schedule for setting out traps, instances of trespassers or other animals having tampered with baited traps, or sperm samples that were lost. The Materials and Methods section indicates the specific sperm parameters and other measures that were recorded, as well as which measures were assessed at the time animals were brought to the mobile lab, and which were assessed at a subsequent time. The specific statistical test used for the paired animal data should be noted (Tables 3 and 4). (Generally, the Shapiro-Wilk test can be used for the testing of normality of datasets. Means and standard deviations for animal body weights, organ-to-body weight ratios, sperm motility and motion data, total count data, and sperm morphology data can be calculated and then analyzed by ANOVA).

The Results section presents two tables of summarized, statistically compared information. Tables 3 and 4, excerpted from an RSA application conducted in the western U.S., provide examples of how body weights and organ-to-body weight ratios, and sperm parameter information are respectively reported. Table 5 (also an excerpt from an actual MRAR) provides a summary of the histopathological analysis that ordinarily accompanies RSA applications. Importantly, histopathological findings are not statistically analyzed but are summarized in a detailed narrative. The following is an example of a detailed narrative.

> No significant differences in the microscopic findings were noted in the liver, spleen, kidneys and, testes between the study areas.
>
> Minimal inflammation (subacute inflammation and leukocytic infiltrate, mononuclear, portal) is a common background finding in the liver of rodents. Similarly, cysts were an incidental finding in two rodents' livers from the Reference areas (Nos. 42 and 51) and are most likely parasitic in origin. The hepatic cyst in animal 51 was characterized by a margin or granulomatous inflammation. Numerous cells with foamy cytoplasm occupied the hepatic cyst margin in animal 42. Although these cells are probably macrophages, the nuclear morphology was not characteristic of macrophages. Pigment laden Kupffer cells were noted in animals from both the Reference and Impact areas with equal frequency. The pigment was light brown and granular reminiscent of hemosiderin. In addition, the liver of one rodent from the Reference area

Table 3. Summary of body weight and organ weight-to-body weight ratio (small animals)

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____ _____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

AREA GROUPING:		Reference Location	Contaminated Site
ANIMAL BODY WEIGHT (BW) [a] (grams)			
	MEAN	22.2	17.9[b]
	SD	4.5	3.1
	N	18	32
LIVER/BW RATIO (% of Body Weight)			
	MEAN	4.47	5.12
	SD	0.70	1.06
	N	18	32
SPLEEN/BW RATIO [a] (% of Body Weight)			
	MEAN	0.1814	0.6015[b]
	SD	0.0486	0.7001
	N	18	32
KIDNEYS/BW RATIO (% of Body Weight)			
	MEAN	1.4690	1.6447[b]
	SD	0.1579	0.2136
	N	18	32
LEFT EPIDIDYMIS/BW RATIO (% of Body Weight)			
	MEAN	0.2175	0.2151
	SD	0.0935	0.0964
	N	18	32
TESTES/BW RATIO (% of Body Weight)			
	MEAN	1.40	1.17
	SD	0.63	0.51
	N	18	31

_____

Notes:

[a] Data are not normally distributed (Shapiro-Wilk Test $p < 0.05$).

[b] Statistically different from Control Group ($p < 0.05$).

Table 4. Summary of sperm analysis parameters (small animals)

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____ _____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

AREA GROUPING:		Reference Location	Contaminated Site
MOTILITY			
(% MOTILE)			
	MEAN	67	35[b]
	SD	27	23
	N	14	24
PROGRESSIVE MOTILITY [a]			
(% PROGRESSIVELY MOTILE)			
	MEAN	58	14[b]
	SD	30	16
	N	14	24
VAP			
(μm/sec)			
	MEAN	126.6	69.5[b]
	SD	34.9	28.2
	N	14	24
VCL			
(μm/sec)			
	MEAN	257.4	142.9[b]
	SD	63.4	49.9
	N	14	24
VSL *			
(μm/sec)			
	MEAN	102.6	50.3[b]
	SD	32.5	24.1
	N	14	24
BCF			
	MEAN	27.2	26.2
	SD	3.6	5.5
	N	14	24
EPIDIDYMAL SPERM COUNT[a]			
(10^6 SPERM/GRAM OF TISSUE)			
	MEAN	1794.0	859.6[b]
	SD	1129.8	848.5
	N	18	31
MORPHOLOGY[a,c]			
(% ABNORMAL SPERM)			
	MEAN	0.3	0.5
	SD	0.5	0.6
	N	16	12

Notes:
[a] Data are not normally distributed (Shapiro-Wilk Test $p < 0.05$).
[b] Statistically different from Control Group ($p < 0.05$).
[c] Mean and standard deviations were calculated using the total number of abnormal sperm as a percentage of the number of sperm examined.

Table 5. Summary of microscopic histopathological findings (small animals)[a]

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____
_____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

AREA GROUPING:	Reference Location	Contaminated Site
NUMBER OF ANIMALS	**18**	**32**
LIVER (EXAMINED)	(18)	(32)
NORMAL	6	16
INFLAMMATION, SUBACUTE	5	6
LEUKOCYTIC INFILTRATE, MONONUCLEAR, PORTAL	5	12
MICROGRANULOMA	1	0
PIGMENT LADEN MACROPHAGES	2	1
GRANULOMATOUS INFLAMMATION	2	0
CYST	2	0
ANISOKARYOSIS	0	1
NECROSIS, HEPATOCELLULAR	1	0
SPLEEN (EXAMINED)	(18)	(32)
NORMAL	10	11
ATROPHY	0	4
LYMPHOID DEPLETION	0	4
LYMPHOID HYPERPLASIA	8	8
RETICULOENDOTHELIAL CELL HYPERPLASIA	0	1
INCREASED EXTRAMEDULLARY HEMATOPOIESIS	0	7
REDUCED EXTRAMEDULLARY HEMATOPOIESIS	2	2
PIGMENT LADEN MACROPHAGES	0	2
KIDNEY (EXAMINED)	(18)	(32)
NORMAL	12	17
BASOPHILIC TUBULES	2	4
TUBULAR DEGENERATION	2	1
TUBULAR ATROPHY	1	1
INFLAMMATION MONONUCLEAR, INTERSTITIAL	5	12
INFLAMMATION SUBACUTE, INTERSTITIAL	2	1
INFLAMMATION, CHRONIC	1	1
LEUKOCYTIC INFILTRATE, MONONUCLEAR, PERIURETERAL	0	2
MINERALIZATION	0	1
TESTES (EXAMINED)	(18)	(32)
NORMAL	9	21
MULTINUCLEATED SPERMATIDS	0	2
TUBULAR DEGENERATION	1	3
IMMATURE TUBULES	8	8
REDUCED SPERMATOGENESIS	2	2
LEUKOCYTIC INFILTRATE, MIXED	0	2

_____
Note:
[a] Incidence of findings not statistically analyzed.

(animal 59) was characterized by locally extensive necrosis accompanied by multifocal chronic inflammation at the necrotic margins. The origin of this lesion is unclear.

Variability in splenic sizes was present in both the Reference and Impact areas. Size differences related predominantly to differences in blood volume, white pulp expansion (lymphoid hyperplasia), and to the degree of hematopoietic cell proliferation (increased EMH). Splenic atrophy resulting from lymphoid depletion was present only in the Impact area. The incidence within the Impact areas was low (~12%). A similar change was not noted in the spleens of Reference area animals. The population of animals in the Reference area was approximately one-half the number in the Impact area group population. Inflammation and degenerative tubular changes of minimal severity are common in rodent kidneys and were present in both Reference and Impact animals.

As discussed in Chapter 2, all information collected in the course of an RSA application other than that which relates to the sperm parameters (e.g., trap success statistics) cannot influence the reproduction-based determination. This is true even if there should be striking differences observed between contaminated site and reference location animals. There are two principal reasons why the other information types do not contribute to the determinations. First, for all features other than sperm parameter shifts, it is not known how much of a measured or observed difference matters in a health assessment context. Second, it would be naive to think that observed differences in such things as histopathology or internal organ size, coincidentally first materialized in the season when the RSA application occurred. Also discussed in Chapter 2, contaminated sites that submit to RSA (and ERAs overall) are typically three or more decades old. At such aged sites, it is likely that any somatic differences noted during an RSA effort have been present for quite some time. The differences were never noted because tasks such as histopathological analysis or organ-to-body weight ratio computation are not routinely conducted tasks in ERA work. Further, on those occasions when rodents are collected, it is usually only done in order to determine (whole) body burden, with this information to be used in food-chain modeling exercises (for carnivorous species that consume rodents). Thus, rodent collections other than for RSA purposes are not oriented to assessing the health of the rodents themselves.

In addition to the above-listed principal reasons for not incorporating nonsperm information into RSA determinations, is the nonreliability of certain field-collected data of RSA applications such as rodent population statistics. Rodent population dynamics cannot be accurately characterized based on data collected during a single trapping event conducted over an approximate 10-day period (the typical duration of an RSA field application). Any number of environmental conditions could account for a great

disparity in the population statistics of a contaminated site and its matched reference location. However, it may be useful to note that past RSA and other ERA field efforts typically find a high parity between contaminated sites and matched reference locations for such things as species numbers, total catch numbers, catch per unit effort, sex ratios, and age distribution. In the absence of sperm parameter-based threshold exceedances for a contaminated site of interest, it does not matter that recorded population ecology statistics at a site (relative to that of the reference location) appear to be far from the ideal; the population statistics have no bearing on any RSA-based reproductive health determination. Importantly, based on historical RSA applications, the nonsperm parameter data gathered in an application are anticipated to serve in a corroborative capacity vis-à-vis the comparative sperm parameter analysis.

Tables 6 and 7 illustrate a useful reporting format for animal and organ weights. Four-decimal place reporting is strongly suggested. The transparency in the reporting of count and motility information in Table 8 enables the user to understand why sample sizes may not be the same for all sperm parameters. The raw data also allows for recomputing population means if the user deems there is justification for extruding one or more animals from a dataset. Also evident are raw data for progressive sperm motility and four sperm motion parameters (i.e., velocity of the average path (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), and beat cross frequency (BCV)). All of this motility information is routinely generated by CASA systems that are in vogue today. Although it has not been definitively shown that progressive motility is as good a barometer of reproductive success as is the conventionally reported 'total' motility, there are indications that it may be so (Tannenbaum and Lee 2010). Table 9 provides the raw data supporting a sperm morphology assessment. In this MRAR-excerpted example, the user can see which specific abnormalities were observed. Sperm morphology is the most sensitive of the three sperm parameters. A nonstatistically significant excess of misshapen sperm of just 4% (relative to control rate) is interpreted to mean that there is reproductive compromise for contaminated site mammals.

Table 10 contains nearly all the information that may be assembled and presented to an ecological risk assessor or risk manager who is fluent with RSA. This table was designed for illustrative purposes only, and it is not recommended that an MRAR include such a compilation. As is first evident, there are no exceedances of sperm parameter-based thresholds for effect. (See the beginning of Chapter 4 for a listing of the thresholds.) In the absence of threshold exceedances, the RSA determination is that the contaminated site in question is protective of not only small rodents, but by extension the larger mammals that also contact the site. The mammalian species collections population data provided in the table (excerpted from Tannenbaum et al. 2003a, reporting on a 2000 RSA application), illustrates several points most relevant to

Table 6. Individual body and organ weight data (all data displayed is in grams)

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____
_____2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

UNIQUE ANIM. ID	ANIMAL ID	ANIMAL WEIGHT	LIVER WEIGHT	SPLEEN WEIGHT	KIDNEY WEIGHT	LEFT EPIDIDYMIS WEIGHT	TESTES WEIGHT
REFERENCE AREA							
42	CA1-P-14	24.0024	0.9024	0.0233	0.2904	0.0402	0.3079
43	CA1-P-19	16.3796	0.7664	0.0264	0.2863	0.0314	0.1892
44	CA1-P-20	26.5551	1.2925	0.0441	0.3904	0.0826	0.5664
45	CA1-P-28	30.4202	1.2941	0.0586	0.4161	0.0937	0.6067
46	CA1-P-44	24.4909	0.9688	0.0356	0.3100	0.0355	0.2781
47	CA1-P-53	27.6936	1.1952	0.0428	0.3828	0.1133	0.6214
48	CA2-P-35	20.8979	0.982	0.0426	0.2963	0.0393	0.1948
49	CA2-P-83	23.8081	1.0429	0.0440	0.3200	0.0775	0.4238
50	CA2-P-3	24.3468	0.9573	0.0297	0.3412	0.0844	0.6185
51	CA3-P-19	23.7028	0.8523	0.0490	0.3837	0.0653	0.4315
52	CA3-P-75	18.9704	0.7485	0.0351	0.2840	0.0189	0.1404
53	CA4-P-87	19.8515	1.2305	0.0442	0.2879	0.0272	0.1778
54	CA4-P-40	17.7151	0.8521	0.0540	0.2821	0.0433	0.2588
55	CA1-P-5	13.4907	0.4986	0.0249	0.2211	0.0083	0.0260
56	CA1-P-51	28.7549	1.1094	0.0311	0.3640	0.0422	0.4084
57	CA1-P-97	19.9755	1.1120	0.0397	0.2883	0.0331	0.1131
58	CA3-P-93	20.6444	0.9828	0.0485	0.3284	0.0393	0.2211
59	CA3-P-72	17.9363	0.9431	0.0344	0.3118	0.0363	0.3233
60 [a]	CA4-P-10	20.7165	0.8880	0.0301	0.2909	0.0306	0.0927
IMPACT AREA							
2	HE1-P-78	19.5870	1.2650	0.0800	0.2830	0.0540	0.3280
3	HE1-P-76	21.6740	1.1120	0.0730	0.3640	0.0800	0.1860
5	HE1-P-29	16.2500	0.6460	0.0520	0.2770	0.0560	0.2050
7	HE2-P-33	16.2220	0.7860	0.0400	0.2050	0.0350	0.1060
10	HE2-P-98	21.7880	1.0440	0.0710	0.3490	0.0800	0.3190
11	HE3-P-12	17.8870	0.5930	0.0330	0.3120	0.0640	0.4000
12	HE3-P-48	15.7200	0.5180	0.0350	0.3040	0.0270	0.1880
14	HE3-P-10	14.7260	0.6120	0.0250	0.2870	0.0110	0.0910
15	HE4-P-6	15.5910	0.7440	0.0390	0.2520	0.0100	0.0990
16	HE4-P-64	17.4130	0.8170	0.0430	0.3700	0.0120	0.1300
17	HE4-P-68	16.0160	0.9860	0.4990	0.3360	0.0260	0.1140
18	HE4-P-51	16.8120	1.0040	0.0490	0.3010	0.0350	0.1350
19	HE4-P-8	15.5850	0.6340	0.0580	0.2010	0.0150	0.1490
20	HE4-P-43	17.1060	0.7890	0.0310	0.2840	0.0220	0.1610
21	HE4-P-40	16.4640	0.9200	0.2470	0.2450	0.0410	0.2180
22	HE4-P-77	16.9480	0.8500	0.0280	0.2630	0.0420	0.1080
24	HE3-P-10	18.8910	1.1490	0.0490	0.3300	0.0600	0.3680
25 [a]	HE1-P-58	15.7480	0.9430	0.0410	0.2110	0.0220	0.0290
26	HE2-P-44	17.8910	0.7090	0.0240	0.2570	0.0380	0.2700
27	HE4-P-12	16.5770	0.9410	0.0660	0.2310	0.0320	0.1410
28	HE4-P-40	22.8070	1.1340	0.2370	0.3450	0.0660	0.4040
29	HE3-P-49	16.6710	0.8690	0.1060	0.2750	0.0260	0.1080

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Note:
[a] Animal immature based on histopathological evaluation of the testis.

Table 7. Individual organ weight-to-body weight ratios

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____
_____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

UNIQ. ANIM. ID	ANIMAL ID	LIVER TO BW RATIO	SPLEEN TO BW RATIO	KIDNEY TO BW RATIO	LEFT EPIDIDYMIS TO BW RATIO	TESTES TO BW RATIO
REFERENCE AREA						
42	CA1-P-14	3.7596	0.0971	1.2099	0.1675	1.2828
43	CA1-P-19	4.6790	0.1612	1.7479	0.1917	1.1551
44	CA1-P-20	4.8672	0.1661	1.4702	0.3111	2.1329
45	CA1-P-28	4.2541	0.1926	1.3678	0.3080	1.9944
46	CA1-P-44	3.9558	0.1454	1.2658	0.1450	1.1355
47	CA1-P-53	4.3158	0.1545	1.3823	0.4091	2.2438
48	CA2-P-35	4.7143	0.2038	1.4178	0.1881	0.9322
9	CA2-P-83	4.3804	0.1848	1.3441	0.3255	1.7801
50	CA2-P-3	3.9319	0.1220	1.4014	0.3467	2.5404
51	CA3-P-19	3.5958	0.2067	1.6188	0.2755	1.8205
52	CA3-P-75	3.9456	0.1850	1.4971	0.0996	0.7401
53	CA4-P-87	6.1985	0.2227	1.4503	0.1370	0.8957
54	CA4-P-40	4.8100	0.3048	1.5924	0.2444	1.4609
55	CA1-P-5	3.6959	0.1846	1.6389	0.0615	0.1927
56	CA1-P-51	3.8581	0.1082	1.2659	0.1468	1.4203
57	CA1-P-97	5.5668	0.1987	1.4433	0.1657	0.5662
58	CA3-P-93	4.7606	0.2349	1.5907	0.1904	1.0710
59	CA3-P-72	5.2581	0.1918	1.7384	0.2024	1.8025
60 [a]	CA4-P-10	4.2864	0.1453	1.4042	0.1477	0.4475
IMPACT AREA						
2	HE1-P-78	6.4584	0.4084	1.4448	0.2757	1.6746
3	HE1-P-76	5.1306	0.3368	1.6794	0.3691	0.8582
5	HE1-P-29	3.9754	0.3200	1.7046	0.3446	1.2615
7	HE2-P-33	4.8453	0.2466	1.2637	0.2158	0.6534
10	HE2-P-98	4.7916	0.3259	1.6018	0.3672	1.4641
11	HE3-P-12	3.3153	0.1845	1.7443	0.3578	2.2363
12	HE3-P-48	3.2952	0.2226	1.9338	0.1718	1.1959
14	HE3-P-10	4.1559	0.1698	1.9489	0.0747	0.6180
15	HE4-P-6	4.7720	0.2501	1.6163	0.0641	0.6350
16	HE4-P-64	4.6919	0.2469	2.1248	0.0689	0.7466
17	HE4-P-68	6.1563	3.1156	2.0979	0.1623	0.7118
18	HE4-P-51	5.9719	0.2915	1.7904	0.2082	0.8030
19	HE4-P-8	4.0680	0.3722	1.2897	0.0962	0.9560
20	HE4-P-43	4.6124	0.1812	1.6602	0.1286	0.9412
21	HE4-P-40	5.5879	1.5002	1.4881	0.2490	1.3241
22	HE4-P-77	5.0153	0.1652	1.5518	0.2478	0.6372
24	HE3-P-10	6.0823	0.2594	1.7469	0.3176	1.9480
25 [a]	HE1-P-58	5.9881	0.2604	1.3399	0.1397	0.1842
26	HE2-P-44	3.9629	0.1341	1.4365	0.2124	1.5091
27	HE4-P-12	5.6765	0.3981	1.3935	0.1930	0.8506
28	HE4-P-40	4.9722	1.0392	1.5127	0.2894	1.7714
29	HE3-P-49	5.2126	0.6358	1.6496	0.1560	0.6478

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Note:
[a] Animal immature based on histopathological evaluation of the testis.

Table 8. Individual sperm motility and total count data

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____ _____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

UNIQUE ANIM. ID	ANIMAL ID	------PERCENT------ MOTILITY	PROGRESSIVE MOTILITY	------MOTION PARAMETERS------ VAP	VCL	VSL	BCV	TOTAL[a] SPERM COUNT
REFERENCE AREA								
42	CA1-P-14	69	53	100	221	77	20	400.4
43	CA1-P-19	34	19	82	172	60	25	2321.1
44	CA1-P-20	96	89	133	296	111	27	2604.3
45	CA1-P-28	91	91	175	350	151	33	3364.8
46	CA1-P-44	40	37	162	287	127	30	914.9
47	CA1-P-53	88	84	164	332	133	29	3397.3
48	CA2-P-35	27	17	90	184	70	27	2232.0
49	CA2-P-83	88	83	132	282	106	26	1323.2
50	CA2-P-3	92	91	178	318	156	33	3691.4
51	CA3-P-19	95	93	148	322	121	28	2150.3
52	CA3-P-75							392.1
53	CA4-P-87							2038.5
54	CA4-P-40	56	41	98	210	76	26	1169.5
55	CA1-P-5		NO SPERM PRESENT FOR ANALYSIS					335.1
56	CA1-P-51	28	24	131	263	111	27	1565.5
57	CA1-P-97		NO SPERM PRESENT FOR ANALYSIS					290.5
58	CA3-P-93	49	25	89	175	68	28	1079.7
59	CA3-P-72	88	66	90	192	70	22	3021.2
60[c]	CA4-P-10		NO SPERM PRESENT FOR ANALYSIS					266.7
IMPACT AREA								
2	HE1-P-78							1242.5
3	HE1-P-76	47	8	59	126	46	26	659.2
5	HE1-P-29	45	10	69	151	44	31	1147.2
7	HE2-P-33	39	15	73	159	50	24	490.1
10	HE2-P-98	30	26	137	230	113	26	351.2
11	HE3-P-12	39	24	92	205	62	24	946.9
12	HE3-P-48	7	1	63	135	36	31	454.6
14	HE3-P-10	29	2	57	128	40	30	256.8
15	HE4-P-6		NO SPERM PRESENT FOR ANALYSIS					[b]
16	HE4-P-64	6	1	66	140	42	24	460.0
17	HE4-P-68		NO SPERM PRESENT FOR ANALYSIS					203.2
18	HE4-P-51	2	0	25	61	18	15	296.0
19	HE4-P-8		NO SPERM PRESENT FOR ANALYSIS					675.7
20	HE4-P-43	4	0	18	44	4	12	388.4
21	HE4-P-40	14	5	78	151	47	24	947.3
22	HE4-P-77		NO SPERM PRESENT FOR ANALYSIS					147.8
24	HE3-P-10	43	22	97	202	61	28	263.7
29	HE3-P-49	1	0	28	51	25	20	28.0
30[c]	HE4-P-28	0	0	0	0	0	0	[b]
36	HE2-P-97		DIED DURING TRANSPORT; NO SPERM OBTAINED					1456.4

Notes:
[a]Million sperm/gram tissue
[b]No epididymis present for analysis.
[c]Animal immature based on histopathological evaluation of the testis.

Table 9. Individual sperm morphology data

REPRODUCTIVE ASSESSMENT FOR INSTALLATION: _____ _____ 2004

ONLY SEXUALLY MATURE ANIMALS (Peromyscus)

UNIQUE ANIMAL ID	ANIMAL ID	Head					Tail			
		Normal	Amorphous	Small	Enlarged	Double	Coiled	Bent	Double	Other
REFERENCE AREA										
42	CA1-P-14	200	0	0	0	0	0	0	0	0
43	CA1-P-19	199	0	0	0	0	0	1	0	0
44	CA1-P-20	200	0	0	0	0	0	0	0	0
45	CA1-P-28	200	0	0	0	0	0	0	0	0
46	CA1-P-44	200	0	0	0	0	0	0	0	0
47	CA1-P-53	200	0	0	0	0	0	0	0	0
48	CA2-P-35	200	0	0	0	0	0	0	0	0
49	CA2-P-83	197	2	0	0	0	0	1	0	0
50	CA2-P-3	200	0	0	0	0	0	0	0	0
51	CA3-P-19	198	2	0	0	0	0	0	0	0
52	CA3-P-75	200	0	0	0	0	0	0	0	0
53	CA4-P-87	198	1	1	0	0	0	0	0	0
54	CA4-P-40	200	0	0	0	0	0	0	0	0
55	CA1-P-5	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
56	CA1-P-51	198	2	0	0	0	0	0	0	0
57	CA1-P-97	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
58	CA3-P-93	199	0	1	0	0	0	0	0	0
59	CA3-P-72	200	0	0	0	0	0	0	0	0
60 [a]	CA4-P-10	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
CONTAMINATED SITE										
2	HE1-P-78	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
3	HE1-P-76	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
5	HE1-P-29	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
7	HE2-P-33	197	1	0	0	1	0	0	1	0
10	HE2-P-98	200	0	0	0	0	0	0	0	0
11	HE3-P-12	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
21	HE4-P-40	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
22	HE4-P-77	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
24	HE3-P-10	200	0	0	0	0	0	0	0	0
26	HE2-P-44	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
32	HE4-P-96	FEWER THAN 200 SPERM CELLS AVAILABLE FOR EVALUATION								
33	HE1-P-59	200	0	0	0	0	0	0	0	0
34	HE2-P-32	198	0	0	0	0	0	1	1	0
35	HE2-P-96	200	0	0	0	0	0	0	0	0
36	HE2-P-97	199	1	0	0	0	0	0	0	0
37	HE3-P-11	197	1	0	0	1	0	0	1	0
38	HE3-P-90	200	0	0	0	0	0	0	0	0
39	HE3-P-83	200	0	0	0	0	0	0	0	0
40	HE4-P-41	200	0	0	0	0	0	0	0	0
41	HE4-P-68	198	2	0	0	0	0	0	0	0

Note:
[a] Animal immature based on Histopathological evaluation of the testis.

Table 10. Cumulative RSA results to consider for a reproductive health determination[a]

Measures	Reference Location	Contaminated Site	delta (Site relative to Reference Location)
Sperm Parameters (population means)			
Count (10^6 sperm/gr.)	1670	1409	-16.71
Motility (% moving sperm)	98.4	99.2	+0.84
Abnormality rate (per 200 cells)	0	0	----
Population data: mammalian species collections			
White-footed mouse	33	29	
Meadow vole	4	22	
Easter cottontail rabbit	2	2	
Deer mouse	0	1	
Masked shrew	0	1	
Short-tailed shrew	17	0	
Eastern chipmunk	36	0	
Meadow jumping mouse	1	1	
Southern flying squirrel	2	1	
Woodland vole	1	0	
Total animals collected	96	55	
Population data: age distribution and sex ratio			
- White-footed mouse:			
adult males	8	7	
adult females	9	5	
sub-adult males	5	3	
sub-adult females	3	3	
juvenile males	3	7	
juvenile females	5	4	
- Meadow vole:			
adult males	1	4	
adult females	1	11	
sub-adult males	0	0	
sub-adult females	0	3	
juvenile males	2	3	
juvenile females	0	1	
Ratio of organ-to-body weight ratios (site/reference location)			
Liver	1.15		
Spleen	3.32		
Kidneys	1.12		
Testes	0.84		
epididymis	0.99		

Note:

[a] Data excerpted from Tannenbaum et al. 2003

interpreting RSA results. First, opportunities to consciously or subconsciously apply bias when reviewing data are always present. Failing to recall that a clear-cut representation of a site's small mammal community cannot be gleaned from a one-time field collection conducted over an approximate 2-week period, one could wrongly conclude that the contaminated site is limited to small mammals based on the total capture numbers (i.e., 96 vs. 55). A more correct review of the tabular information shows that there are more instances of parity for the two sites than there are of differences. Both sites have nearly the same number of individuals of the dominant species (*Peromyscus sp.*) (i.e., the lone species that submitted to a sperm parameter analysis). Both sites also have nearly identical numbers of six occasional species. Failing to recall that animal capture information cannot trump the sperm parameter analysis, yet to show that the contaminated site cannot well support mammals, a reviewer might harp on the presence of chipmunks only at the reference location. In doing so, however, the reviewer would be overlooking a trend in the opposite direction, albeit one that is not quite as dramatic. There were many more meadow voles at the contaminated site than there were at the reference location, something worthwhile noting, given that the meadow vole has been the second most commonly evaluated species in RSA.

Table 10 also illustrates the reality that highly matched reference locations are truly hard to find. Although the two sites had highly similar vegetative cover (including numbers of trees), soil type, and slope, on subsequent review, it was discovered that most trees at the reference location were nut trees, quite unlike the contaminated site. With the chipmunk's dominant dietary item being nuts, this difference in tree type very likely accounts for the dichotomy observed with regard to chipmunk presence.

Population data should be reviewed for its potential to provide corroborative support to sperm parameter-based determinations. Table 10's age distribution and sex ratio population data is helpful. For the White-footed mouse, the sex ratio and age distribution are nearly the same for the site and reference. Barring some other overlooked habitat distinction (akin to the difference in the number of nut trees at the two sites), overall meadow vole numbers, sex ratio and age distribution, should all be similar for the two sites. As per the table though, voles at the contaminated site outnumber voles at the reference location in every category.

Azoospermia is unlikely to arise in RSA datasets (see end of Chapter 4). Should it be present though, the procedure to invoke involves discounting those rodents that had no sperm, and constructing the so-called "site population" sperm count average (as well as the "site population" average sperm motility and morphology) using only those rodents that *did* have sperm. An explanation for this scenario is provided here because proceeding this way may seem to be counter-intuitive.

The purpose in constructing an average sperm count for a given parcel, contaminated or clean, is to have a representative expression of the reproductive fitness (sperm titer) for what should be the typical adult male rodent with which any given female might mate (Tannenbaum et al. 2008). Male rodents that do not manufacture sperm are incapable of influencing this reproductive fitness expression. These male rodents might attempt to exhibit copulatory behavior, but these so-called 'matings' will not lead to fertilizations (and litters subsequently being born). Although copulating azoospermic males occupy females' time that could otherwise be more appropriately utilized for engaging in copulatory behavior with fertile males, it can be assumed that the promiscuous behavior of rodents will ensure that females also copulate with fertile males. Importantly, averaging in zero counts along with positive sperm counts would have the effect of producing misleading estimates of male reproductive capability. In actuality, there is no physical mixing of batches of sperm or semen of different animals taking place either in some hypothetical reservoir or within the reproductive tract of a sexually active female rodent. Should a female mate with an azoospermic male, either before or after copulating with a fertile male, there will be no sperm titer reduction as a result of this (Tannenbaum et al. 2008).

Table 2 not only demonstrates the computation of an average sperm count for a contaminated site with the aberration of azoospermia, it shows that for the specific RSA case profiled the reproductive capability of the contaminated site is actually enhanced over that of the reference location. The example illustrates that although an aberration (i.e., a male population being 75% azoospermic) needs to be documented in the MRAR, the aberration has no bearing on the RSA determination. Population ecology routinely assumes that there is a sufficiency of males to inseminate females. (In this actual example, the four fertile males are assumed to be responsible for having sired all of the Hispid cotton rats in the study area during the given year or season when the RSA application occurred.) Although there may be an interest (on the part of stakeholders) in computing the percentage of males that are azoospermic within a population, such information cannot be of assistance within an RSA context. It is not known how great this percentage needs to be to pose reproductive challenges to the population, if it should pose any at all. An excerpt from Tannenbaum et al. 2008 that discusses an actual occurrence of azoospermia in an RSA application conducted in south-central U.S. is relevant to this chapter's discussion on biases that can interfere with RSA data interpretation.

> Our unusual finding highlights the enormous potential that exists for stakeholders, and in particular regulators, to draw errant conclusions in field-based terrestrial ERA. In this instance, had the extent of the field assessment work amounted to no more than a comparative rodent trapping success effort, stakeholders would likely have all agreed that the local rodent population is

stable and seemingly healthy. The uncertainties associated with trapping animals over a brief 2-week window would, of course, have been acknowledged. It is curious that stakeholders could draw such a conclusion without the luxury of any somatic information (e.g., organ weights, histopathology, and enzyme levels). It is still more curious that at the first sign of a somatic measure difference between site and reference location animals, as in the discovered azoospermia at the (contaminated) site, the animal capture information would then be seen to hold little if any value. Such is the context in which biological information is scrutinized in ERA today. Collecting comparative biological measures, although well intended, runs the risk of having stakeholders interpret any and all observed differences as problematic (Tannenbaum 2001). By way of example, a frequently encountered difficulty is that stakeholders will interpret a somatic difference (e.g., an altered liver enzyme level) observed at a multiple decade-old contaminated site, as reflecting a biological response that only recently happened. As with the azoospermia we observed at the contaminated site, we believe it to be too coincidental that such changes arise for the very first time, coincident with a field investigation. In our case, it is unrealistic to think that the azoospermia we observed in our Hispids first arose in the year that the RSA field effort was conducted. Given the overly cautious mindset of stakeholders, it might be thought prudent to deliberately plan for not collecting any somatic information in field-based ERA efforts. For stakeholders to be able to agree that site receptors are not being harmed at a contaminated site though, a concrete biological measure that can demonstrate receptor health is very much needed.

CHAPTER 6
SAFETY

Prior to conducting RSA in the field, a health and safety plan (HSP) should be published and approved by the Army interest/customer (e.g., installation) requesting the work. At a minimum, the HSP should identify existing site-specific hazards (e.g., potential for snake bites, presence of Poison ivy). The installation should be contacted to gather such information. The preliminary site visit (Chapter 3) is an opportune time to observe first-hand the dangers and hazards that can arise. An absolute requirement for the HSP is that a nearest hospital or medical care facility be identified along with clear driving instructions to arrive there from the installation (see Chapter 3).

The most serious potential danger to individuals involved with RSA duties is that of becoming infected with Hantavirus. Many rodent species captured in the wild, even at locations far removed from areas in the U.S. where the virus is known or expected to occur, may carry the virus. With opportunities to become infected through handling rodents (especially when there are breaks in the skin on the hands), certain safety procedures must be implemented. All individuals checking live animal traps for captures must wear gloves and must hold the trap downwind at arm's length. Traps with adult male rodents to be assessed must be conveyed to the onsite mobile laboratory by truck (and not car) with the traps in a separate compartment from the driver and any other passengers. Individuals euthanizing rodents and doing dissections (e.g., harvesting organs) must wear surgical gloves and conduct all such work under a fume hood. Alternatively, a powered air-purifying respirator unit may be worn when conducting such activities.

Animal carcasses and remains, other than those that are to be preserved for species identification, must be disposed of in red plastic biomedical waste bags at a facility licensed to handle such waste. Animals to be identified to species should be kept frozen in clearly labeled plastic bags until they are turned over to an institution of choice for mammalogists to examine the specimens.

Disposable scalpel blades must be thrown away in a biomedical waste (sharps) container. Surgical tools (forceps, scissors) must be cleaned with Alconox® or a similar biocide after each use. (Alconox® is a registered trademark of Alconox, Inc.)

CHAPTER 7
STATUS OF THE RODENT SPERM ANALYSIS METHOD

The RSA method, first applied in 2000, was patented in December 2009. The RSA method is unique in that it appears to be the only patented method in the health risk assessment arena (comprised of both HHRA and ERA). The Army pursued a patent for RSA for two reasons. It recognized the method as a scientific tool that advances the science of ERA in the realm of arriving at near-definitive health determinations for ecological receptors. The Army also recognized the potential for considerable cost savings when there is a credible line-of-evidence in support of site ecological receptors not bearing any ill effects from their exposures at contaminated sites. As mentioned previously, there is a great potential for RSA applications to conclude that ecological health risk concerns are not present at contaminated Army properties. The Army is open to securing licensing agreements from parties interested in applying RSA.

RSA has achieved certain notoriety both before the patent was secured and since then. In 2004, the Army was invited to present the method at one of the Smithsonian Institution's Department of Mammalogy seminars. Additionally, the method has been published in the peer-reviewed literature on four occasions, and has been referenced by others who publish in the peer-reviewed literature. RSA is also prominently mentioned in the Encyclopedia of Ecology (within the heading "Ecotoxicology: Reproductive Toxicity," Tannenbaum 2008). In 2011, the Army provided a formal RSA presentation (webinar format) that was favorably received by the USEPA's Ecological Risk Assessment Forum. Pursuant to that presentation, EPA expressed a willingness to have RSA run at sites where the agency is conducting its own ERA-related field testing.

The Army has always promoted RSA as a method to be run in tandem with conventional desktop-based ERAs, or as a method to be run pursuant to a conventional ERA that found one or more HQs to exceed unity. (In the latter case there is a need to conduct more site-specific work to validate the concerns raised over the HQs which are only crude screening tools.) It is possible though, for the Army to apply RSA at a terrestrial site where a conventional desktop ERA has never been conducted. This opportunity presents itself if: a) a given site is not governed by either Superfund or the Resource Conservation and Recovery Act programs of the USEPA., and b) it is recognized that nearly all terrestrial site HQ-based assessments find that at least one mammal is potentially at risk. With most conventional assessments resulting in a need for additional study, RSA's capacity to arrive at as definitive determinations as are possible should be recognized.

RSA remains a method relegated to ERA (although as explained earlier, it is not itself a risk assessment method). Nevertheless, the potential exists for RSA to be employed to augment HHRAs. Here, RSA would be applied in the standard fashion, after which there would be cautious extrapolation of the results to draw technically sound inferences about potential reproductive effects in certain exposed human populations (e.g., Soldiers). Such an application of the RSA method would fully acknowledge that the rodents involved consistently reflect multi-generational exposures, whereas the human population(s) to be assessed would ordinarily have vastly shorter exposures. An exception to this design, and one that would seemingly constitute the best fit of RSA within an HHRA context, would be where the Army assesses the prospect of human habitability of a presently non-occupied terrestrial property.

CHAPTER 8
FREQUENTLY ASKED QUESTIONS

Since RSA's creation in 2000, numerous tutorials and other reviews of the method have been presented at a wide array of formal and informal venues. A decade after RSA's entry into the ERA arena, the method continues to prompt a small group of questions. The more recurrent questions are addressed in this chapter.

FAQ #1. RSA deals only with males. How can the method be reliably used if it does not deal with a site's female rodent population?

Response: There are several components to a proper answer to this question. First it should be noted that in conventional ERA, computed HQs are often predicated on laboratory studies that involved laboratory mice or rats of just one sex. As well, 96% of all the reproductive studies considered in developing ecological soil screening levels http://www.epa.gov/ecotox/ecossl/index.html; Eco-SSLs) for mammals are one-sex studies. Regarding evaluative efforts that rely on toxicological responses to contaminants, RSA should not be held to a higher standard than conventional ERA; if single-sex studies can support desktop ERAs to the point where the intent is to invoke remedial action based on HQs above 1.0, male-only based RSA outcomes that come far closer to depicting the actual site condition than desktop efforts, should also be able to decide the remedial action question. A second component to the question's answer invokes common sense. If, as per an RSA outcome, a site is deemed to be protective of the reproductive health of its male field rodents, and numerous rodents of both sexes were field trapped (as is seemingly always the case), it must be that there are reproductively capable female rodents at the site to account for the animals observed. A third answer component is that there is precedent for extrapolating reproductive toxicity response in one sex to the other sex (USEPA 1996). In brief, a reproductive toxicity response evoked in one sex of a species is assumed to occur in the other sex. The reader is cautioned to understand that the reproduction-based response spoken of here is a percentage reduction in reproductive system capability (e.g., a 10% compromise in reproductive function).

FAQ #2. Can small rodent-based RSA outcomes be extrapolated to address other (i.e., larger, wider-ranging, and often higher trophic level) mammals?

Response: There is a dual response for this question. First, in present-day ERA we routinely employ the extrapolation of laboratory mouse and rat data to other mammals by adapting the toxicity thresholds of these lab species to those animals for which we

could conceivably invoke a cleanup to afford them greater protection. (Sites are not remediated in order to afford protection to small rodent species.) Thus, if we are already routinely applying such an extrapolation scheme for hypothetical species, it could only be more appropriate to extrapolate the biological condition of actual rodents at contaminated sites to other 'actual' mammals at nearby sites. Second, USEPA guidance clearly supports the extrapolation type asked of here. Effects of xenobiotics on male and female reproductive processes are assumed to be similar across species unless demonstrated otherwise (USEPA 1996).

FAQ #3. RSA speaks to only one of the two terrestrial animal groupings that ERAs target, namely mammals. Although RSA's utility is recognized, is there an equivalent direct health status assessment method for birds (the other animal grouping of concern in terrestrial ERA)?

Response: It is important to recall that RSA's attention to mammals only is a deliberate design feature and not a shortcoming. In appreciating the method's utility, it is important to understand that other than the Army's RSA invention, no other ERA practitioners have dealt with the prospect of directly assessing the health status of (actual) site receptors, regardless of animal grouping. Thus, no assessment scheme isomorphic to RSA exists for birds. There is though a way to apply RSA theory to draw inferences, albeit weaker ones, to birds. No Observed Adverse Effect Levels (NOAEL) and Lowest Observed Adverse Effect Levels (LOAEL) developed from the dominant sources of ecotoxicological benchmarks (such as Sample et al. 1996) consistently show for nearly all metals and quite a few organic compounds, that these toxicological thresholds are more stringently set for mammals (than birds). Where sperm parameter thresholds of an RSA application were not exceeded (indicating that mammals are not health-compromised), we *could* also know that site birds are probably not experiencing health impacts. (Note that interclass extrapolation of toxicological benchmarks, a rather universally contraindicated practice in ERA, is not being implemented.) With mammal effects absent where the sensitive RSA method was run, and with birds being (generally) less sensitive to contaminants, one could conclude that the good health determination for mammals confers a similar designation for birds. By way of example, the respective NOAELs for mammals and birds, for PCB-1242 are 0.069 milligrams per kilogram per day (mg/kg-day) and 0.41 mg/kg-day, and the respective LOAELs for mammals and birds, for PCB-1254 are 0.68 mg/kg-day and 1.8 mg/kg-day.

FAQ #5. How was the number 15 as the "desirable number" of adult male rodents to be sought at contaminated sites and their matched reference locations determined?

Response: There are multiple bases for the number 15. First, this is the number of animals that are routinely used in USEPA laboratory rodent test trials. Second, it is not the intention to decimate rodent populations while conducting RSA. Removing more than 15 adult males at areas at which trapping grids are placed could severely impact local reproduction. Third, site-specific conditions (e.g., locale, species-specific densities) might not allow for as many as 15 adult males to be trapped over a gridded area. Fifteen adult males then is not an absolute number to secure. With regard to the concern over depleting rodent populations where animal trapping occurs, the potential value-added with lesser capture numbers (e.g., 8-10) should not be overlooked. While there may be a tendency to think that there is now data insufficiency, the lesser captures may be reflecting that the entire male population for the site of interest has been sampled (or a figure very close to this)! Finally, a sample size of 15 animals provides assurance that for the most important of the three sperm parameters tracked with RSA (i.e., sperm count), the threshold-for-effect of a 60% count reduction relative to the condition for the reference location can be detected. A review of all RSA datasets reveals that with 15 adult males, a 60% reduction in sperm count relative to the reference location animals can be detected with statistical power ranging from 80 to 99%. A review of all RSA datasets also reveals that detecting a 60% reduction in sperm count with a statistical power of 80% can be achieved with as few as three to four animals. If after a week's worth of trapping, less than three adult males of a given species have been captured, all future captures of adult males of that species should be released to the field at the point of capture, recognizing that there will likely be an insufficient number of target animals collected to allow for a valid comparative sperm parameter analysis.

FAQ #5. Has RSA been endorsed by any of the regulatory agencies?

Currently, there are no formal endorsements in place. On a number of occasions though, regulatory agencies have welcomed the inclusion of RSA to the suite of study approaches employed for a site-specific assessment. It is important to understand that this FAQ may not be a fair one. Regulators may be completely unfamiliar with RSA and may also be leery of its claims. Additionally, regulators involved in site-specific work cannot be expected to embrace a method (such as RSA) that is not part of the status quo. Further, opportunities do not frequently arise whereby a regulator can become fully versed in the RSA method. Even if the regulator does become fully versed in the method, he or she may opt to disagree with it for it overtly breaks with traditional ERA approaches in two key ways: RSA sees the need for direct health status assessment of

the site receptor more so than it sees a need to forecast health effects, and RSA understands that all assessments really only need to determine if reproductive impacts have occurred.

FAQ #6. How much does it cost to run RSA? How do RSA and conventional ERA costs compare?

Critical to answering this question is understanding that the two products do not yield the same information. A conventional ERA is almost never definitive, because almost always one or more site receptors will have HQs greater than 1.0 (thereby necessitating more work). RSA outcomes by nature are always definitive and never recommend follow-on work. In a sense then, the comparison asked about is an 'apples & oranges' one. Where an RSA application is found to cost more than a conventional ERA (an unlikely occurrence), one needs to recall that such a comparison overlooks the minimal gains brought on by the latter. A conventional ERA provides the customer with a two to three volume report that includes the site description and history, an account of the relevant sampling that occurred, a 'contaminants of potential concern' screening exercise, an HQ computational exercise for animals that were never handled (let alone observed), and a summary/conclusions section that discusses unitless HQs that serve only as screening tools. The MRAR of an RSA effort reports on the reproductive status of the very animals that are present at the site (i.e., an assessment of the toxicological endpoint of greatest interest for the maximally-exposed site mammalian receptor). As mentioned in the response to FAQ #5, RSA recognizes that ecological health concerns at contaminated sites consistently reduce to a need to know if reproduction in site receptors has been compromised.

It is hard to assign a price to a contractor-produced conventional ERA for a terrestrial site given the variations in complexity among sites. Still, in order to facilitate the comparison asked about, a reasonable figure might be $75-100K (subject, of course, to economic trends). An RSA effort culminating in an MRAR, like a conventional ERA report, can assume a range of costs. The variables contributing most to this spread are the inclusion/exclusion of: a) the sperm motility measure (discussed in Chapter 3 under "Data Collection"), and b) a histopathological analysis of certain organs. Lesser contributors to the range of costs are travel considerations and length of stay in the field (generally a function of how abundant the small rodent populations of interest are). In providing a workable RSA cost estimate, it is fair to say that the cost would never be expected to exceed $90K. Where the customer opts to dispense with the sperm motility analysis (as is more commonly done as of the time of this writing), and where only 4-5 trapping days are needed (as opposed to 7-10 days), the cost could be in the $50-60K range.

APPENDIX
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