

PROPOSED UPDATES TO THE AAFCO DOG AND CAT FOOD FEEDING PROTOCOLS

RATIONALE FOR CHANGES TO THE AAFCCO DOG AND CAT FOOD FEEDING PROTOCOLS

The proposed changes made by the Feeding Protocols Expert Subcommittee (FPES) are identified throughout the proposed updates to the AAFCO Dog and Cat Food Feeding Protocols, using underlined text to indicate text to be added and ~~struck through text~~ to indicate text to be deleted. A few changes are editorial without substantive effect on how the protocols are performed or interpreted. The remaining proposed changes are substantive. The FPES felt these substantive changes were needed in order to unequivocally indicate how a specific aspect of a protocol was to be accomplished and to ensure the validity of the protocols for establishing nutritional adequacy of products. All substantive changes, as well as some of the editorial changes, are discussed and justified below.

A paragraph was added to the beginning of the AAFCO Dog and Cat Food Feeding Protocols to clearly indicate that successful passage of a protocol validates the nutritional adequacy of the tested product's ingredient formula and the resulting nutrient profile for the species and life stage(s) to which the product was fed. The paragraph adds the clarification that for the claim to be valid, the nutrient profile of the product as formulated and tested should remain stable through the end of the product's expected shelf life.

EDITORIAL CHANGES

A sentence was inserted in the paragraph titled "DOGS" in the MINIMUM FEEDING PROTOCOL FOR PROVING AN ADULT MAINTENANCE CLAIM FOR A DOG FOOD to clearly indicate that historical colony averages must be determined from animals in the testing facility and that those animals must accurately represent the size and breed of the animals in the test group. An identical sentence was also inserted in the paragraph describing the animals (i.e., CATS, PUPPIES, KITTENS) in each of the subsequent protocols for the same reason and consistency.

The outline format used in certain sections throughout the protocols was standardized for consistency to that used in other sections. The format is: uppercase letter followed by a period, alphanumeric number followed by a period, lowercase letter followed by a period (i.e.,

- A.
- B.
 - 1.
 - 2.
 - a.
 - b.)

The first instance of such a revision in formatting occurs in the section titled CLINICAL OBSERVATIONS AND MEASUREMENTS in the MINIMUM FEEDING PROTOCOL FOR PROVING AN ADULT MAINTENANCE CLAIM FOR A DOG FOOD. Similar revisions for the same purpose occur in other sections throughout the protocols.

SUBSTANTIVE CHANGES

The current AAFCO Feeding Protocols state, "Daily food consumption may be measured and recorded." As written, this statement makes any measurement and record of daily food consumption optional; it may be, but does not have to be, measured and recorded. Furthermore, the daily food consumption does not have to be assignable to an individual animal. The FPES believes that any well-conducted feeding protocol would have individual daily food consumption measured and recorded as basic,

standard data collected during performance of a feeding protocol. The FPES considered that some facilities may feed animals as groups rather than individuals, but also notes that the protocols allow for individual animals to be removed during the first two weeks of the study “for non-nutritional reasons or poor food intake.” As a compromise to requiring that individual daily food consumption must be measured and recorded under any and all conditions, the FPES proposes that if the option of removing animals for poor food intake is to be exercised, then poor food intake of the individuals removed from the study must be documented and thus individual food intake must be measured and recorded. To state the condition another way, the language inserted into criterion A. of the CLINICAL OBSERVATIONS AND MEASUREMENTS section of each protocol effectively causes the option of removing animals for poor food intake to be forfeited if individual food consumption is not, or cannot, be measured and recorded.

**MINIMUM FEEDING PROTOCOL
FOR PROVING AN UNQUALIFIED REPRESENTATION
OF NUTRITIONAL ADEQUACY
FOR A DOG OR CAT FOOD**

The words “an unqualified claim for” were added to the first sentence of the first paragraph in the section titled MINIMUM FEEDING PROTOCOL FOR PROVING AN UNQUALIFIED REPRESENTATION OF NUTRITIONAL ADEQUACY FOR A DOG OR CAT FOOD to indicate what type of nutritional adequacy claim the “minimum testing” was substantiating. As previously worded, it could be argued that the minimum testing necessary to prove nutritional adequacy was something less extensive than sequential gestation/lactation and growth protocols. The word “weaning” was inserted into the last sentence of the paragraph because a lag period of several days to weeks could occur between weaning and the beginning of a growth protocol. The FPES deemed that test animals used in the growth protocol should receive only the test diet during any intermittent lag between the end of lactation and the beginning of growth protocols when the protocols were run sequentially for establishment of an unqualified (i.e., an All Life Stages) claim.

**Probabilities, Standard Errors and Allowance for Normal Variation for INTERPRETATION of
Protocol Measurements**

The FPES considered changing the probability of committing a type I error, plus the associated parameter values for standard error and allowance for normal variation, to 0.05 (5%) and associated values for normally distributed data with a sample size of $n=8$. A Type I error is judging the values for a nutritional indicator parameter in a control versus a test group to be different when in fact they are not. For a given sample size, the probability of committing a type I error is inversely proportional to the probability of committing a type II error. A Type II error is not judging the values for a nutritional indicator parameter in a control versus a test group to be different, when in fact they are different. A smaller probability, or likelihood, of a type I error, increases the probability, or likelihood, of concluding that the test diet is equal to the control diet when, in fact, it is not. This means the test diet passes the protocol and is certified as nutritionally adequate when it is not adequate.

However, during full committee review of the proposed changes, historical knowledge revealed that a probability of 0.05 had initially been used when the protocols were established but appeared overly strict with few products passing the protocols to which the products were subjected. A reexamination noted that, particularly for blood parameters, multiple comparisons were being performed and that some control for committing Type I errors was required. Given that there were 4 blood parameters in dogs (hemoglobin, packed cell volume, albumin, alkaline phosphatase) and 5 in cats (hemoglobin, packed cell volume, taurine, albumin, alkaline phosphatase) being determined on one sample, a Bonferroni correction was applied that lowered the value to 0.01 (1%) for each individual comparison and an approximate value of 0.05% for all combined comparisons of the blood parameters.

The FPES noted that for comparison of body weight and weight gain the probability was set at 0.05 for adult maintenance protocols, but at 0.01 for mothers and offspring in the growth and gestation/lactation

protocols. Comparison of litter size was also set at 0.01 in gestation/lactation protocols. The FPES returned the probability for committing a type I error to the generally used value of 0.05 (5%) for comparisons of body weights in all protocols and litter sizes in gestation/lactation protocols, but retained the probability of 0.01 (1%) for comparison of blood parameters.

**MINIMUM FEEDING PROTOCOL FOR PROVING A GROWTH
CLAIM FOR A DOG FOOD
(GROWTH - DOG)**

Because there are documented differences in the rate and magnitude of increased body weight between growing male and female dogs within the same breed, the FPES inserted additional language in the PUPPIES section. The proposed changes now require that historical colony averages for weight gain must be determined for each sex. The colony average for weight gain of male puppies must be determined using a minimum of 30 male puppies and similarly a minimum of 30 female puppies to determine the colony average for weight gain of female puppies. This is in addition to the previously discussed specification that historical colony averages be acquired from a similar population of animals within the same testing facility accurately representing the size and breed of the test group. Unlike weight gain, the other measured parameters specified in the protocol have not been shown to be gender dependent, so historical colony averages for parameters other than weight gain can be determined from a minimum of 30 individual puppies. All of the data for all of the non-weight gain parameters must come from the same puppies. The remaining changes in the PUPPIES section were made for clarity and readability.

In addition to the previously noted changes in the statistical values for weight gain comparisons, the FPES increased the weight gain criterion in C.1 for passing the GROWTH – DOG protocol from 75% to 80% of the historical colony average. The FPES notes that for cats the criterion has been 80% and sees no reason why the criterion for puppies should be less than that for cats.

Given that gender is a factor in the amount of weight gained during growth, the influence of gender must be accounted for in the statistical evaluation of weight gain and the mathematic equations for doing so are slightly more complex than for comparison of other parameters. Therefore, the FPES created two appendices that contain, respectively, the formulas for calculating the adjusted historical colony average and standard error used in criteria C.2, and the average weight gain and normal variation from the concurrent control group used in criteria C.3 for evaluating weight gain. Reference is made to the appropriate appendix in each of the protocols where weight gained of growing animals is a parameter evaluated for documenting nutritional adequacy of the test diet. As discussed above, the factors used in calculating the standard error and normal variation have been adjusted for weight gain to those associated with a probability of 0.05 for committing a type I error with n=8 subjects per group.

**MINIMUM FEEDING PROTOCOL FOR PROVING A GROWTH
CLAIM FOR A CAT FOOD
(GROWTH - CAT)**

The same changes discussed above for the GROWTH - DOG protocol were made in the GROWTH - CAT protocol other than the change for INTERPRETATION criterion C.1 which was already at 80% of the historical colony average.

**MINIMUM FEEDING PROTOCOL FOR PROVING A
GESTATION/LACTATION CLAIM FOR A DOG FOOD
(GESTATION/LACTATION - DOG)**

In addition to changes indicated and discussed above, the FPES deleted the references to normal litter size and the allowance for redistributing puppies among bitches of the same breed with smaller litters based on lack of practical applicability. Similar to the increase from 75% to 80% of the historical colony average

for average body weight in INTERPRETATION C.1 section of the GROWTH – DOG protocol, and for the same reasons, the FPES increased the criterion in D.1 of the INTERPRETATION section of the GESTATION/LACTATION - DOG protocol to 80% of the historical colony average for average body weight of puppies at the end of lactation.

MINIMUM FEEDING PROTOCOL FOR PROVING A GESTATION/LACTATION CLAIM FOR A CAT FOOD (GESTATION/LACTATION - CAT)

All of the changes that occur in the GESTATION/LACTATION - CAT protocol also occur in other feeding protocols and have been discussed above. The FPES proposes to delete the provision for removing kittens from queens with litters larger than five kittens for similar reasons for removing the like provision in the GESTATION/LACTATION – DOG protocol.

CRITERIA FOR SUBSTANTIATION OF CONTINUED VALIDITY OF NUTRITIONAL ADEQUACY BASED ON FEEDING PROTOCOL RESULTS

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The FPES established a new section and requirement to address formula stability and continued validity of feeding protocol-based nutritional adequacy claims.

For the purposes of describing the criteria for substantiating continued validity of nutritional adequacy of products based on feeding protocol results, the FPES defined a “protocol substantiated formula.” As stated in the new paragraph added to the beginning of the AAFCO DOG AND CAT FOOD FEEDING PROTOCOLS, a feeding protocol validates the nutritional adequacy of the tested product’s ingredient formula and the resulting nutrient profile for the species and life stage(s) to which the product was fed. The FPES specified formula changes that would require re-substantiation of nutritional adequacy using either a newly performed feeding protocol or demonstration that the resulting formula meets the criteria of being a Pet Food Product Family member formula with the “protocol substantiated formula” being the lead member of the Pet Food Product Family.

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Because nutrient composition may be altered over time by many factors, including ingredient sources or changes in processing techniques, the FPES felt it prudent to establish criteria for substantiating continued validity of nutritional adequacy. A formula established as stable confirms the continued validity of nutritional adequacy of products based on feeding protocol results. This is to be done every five years for protocol substantiated formulas and Pet Food Product Family members.

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For protocol substantiated formulas, continued validity of nutritional adequacy can be demonstrated either by: re-performing the feeding protocol; or by demonstrating that marketed protocol substantiated formula(s) bearing a feeding protocol substantiated claim for nutritional adequacy contain at least 95% of each of the key nutrients used for establishment of a family member product compared to the content of these nutrients in the product used in the original feeding protocol(s). Monte Carlo simulations using check sample data for estimating variances of the 6 nutrients used for dog foods, or 8 nutrients used for cat foods, to establish Pet Food Product Families predict that 95% of the products should be capable of meeting the criteria for successful demonstration of product stability. Pet Food Family member products must demonstrate the currently marketed product still meets the criteria for being considered a Pet Food Product Family member compared to data from the original “protocol substantiated formula” being used as the reference nutrient values of the lead member of the Pet Food Product Family.

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The proposed revisions to the AAFCO Dog and Cat Food Feeding Protocols are attached below using underline for added text and strikethrough for deleted text.

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AAFCO DOG AND CAT FOOD FEEDING PROTOCOLS

A successfully completed feeding protocol validates the nutritional adequacy of the product's ingredient formula and resulting nutrient profile for the species and life stage(s) to which the product was fed. For the nutritional adequacy claim to be valid, it is expected that the nutrient delivery of the product will not be significantly degraded over the shelf life of the product.

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MINIMUM FEEDING PROTOCOL FOR PROVING AN UNQUALIFIED REPRESENTATION OF NUTRITIONAL ADEQUACY FOR A DOG OR CAT FOOD

The minimum testing necessary to prove an unqualified claim for nutritional adequacy may be obtained by using the gestation/lactation and the growth protocols. These protocols must be used sequentially. Thus, a manufacturer desiring to prove an unqualified claim for nutritional adequacy must use the litters obtained from performing the gestation/lactation protocol for the growth period. Test puppies or kittens shall receive the test diet as their sole source of nourishment, other than dam's or queen's milk, during lactation, weaning, and growth.

Selection of puppies or kittens shall be on a statistically sound basis from each of the litters qualifying for the gestation/lactation protocol with equal sex distribution preferred.

MINIMUM FEEDING PROTOCOL FOR PROVING AN ADULT MAINTENANCE CLAIM FOR A DOG FOOD

DOGS

A minimum of eight healthy adult dogs at least one year of age and of optimal body weight shall be required to start the test. Bitches in gestation or lactation shall be excluded. All animals starting the test must pass an initial physical examination by a veterinarian. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. A minimum of 30 dogs shall be required for developing a historical colony average, with data used to establish averages for all parameters coming from the same individual dogs. A minimum of eight dogs shall be required for the concurrent control group. Breed distribution shall be similar in all groups.

DIET

The same formulation shall be fed throughout the test although different production batches may be used. Diets fed to a concurrent control group or to dogs in the determination of historical colony averages must have successfully passed the minimum feeding protocol for an adult maintenance claim for a dog food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall run for a minimum of 26 weeks and shall begin when dogs are placed on the test diet.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Dogs shall be fed *ad libitum* or based on energy needs. Fresh water shall be provided *ad libitum*. Any interruption in the feeding protocol must be disclosed and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1-~~A. Individual ~~D~~daily food consumption ~~may~~ shall be measured and recorded for all animals if any animal is removed for poor food intake.
- ~~2-~~B. Individual body weights shall be measured and recorded at the beginning, weekly, and at the end of the test.
- ~~3-~~C. Hemoglobin, packed cell volume, serum alkaline phosphatase and serum albumin shall be measured and recorded at the end of the test.

- ~~4-D.~~ All dogs shall be given a complete physical examination by a veterinarian at the beginning and at the end of the test. Each dog shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.
- ~~5-E.~~ Any medication and the reason for its use must be recorded.
- ~~6-F.~~ A number of dogs, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Dogs may be removed for poor food intake only during the first two weeks of the test. Data already collected from dogs removed from the test shall be retained although it does not have to be included in the final results.
- ~~7-G.~~ A necropsy shall be conducted on any dog which dies during the test and the findings recorded.

INTERPRETATION

- A. The diet shall fail if any dog shows clinical or pathological signs of nutritional deficiency or excess.
- B. All dogs not removed for non-nutritional reasons or poor food intake must successfully finish the test.
- C. No individual dog shall lose more than 15% of its initial body weight. The average percent body weight change (final compared to initial) shall not be less than either:
1. Negative ten percent; or
 2. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.05$, if $n=8$ per group).
- D. The average final hemoglobin, packed cell volume and serum albumin values shall not be less than either:
1. a. Hemoglobin - 14.0 g/dl (no individual <12.0 g/dl),
b. PCV - 42% (no individual $<36\%$),
c. Albumin - 2.8 g/dl (no individual <2.4 g/dl); or
 2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).
- E. The average final serum alkaline phosphatase value shall not be greater than either:
1. 150 IU/L (no individual >300 IU/L); or
 2. The historical colony average plus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, plus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).

OPTIONAL PROCEDURES

The testing requirements for a maintenance dog food may be met by successfully performing either the growth or gestation/lactation protocols in lieu of performing the maintenance protocol.

MINIMUM FEEDING PROTOCOL FOR PROVING AN ADULT MAINTENANCE CLAIM FOR A CAT FOOD

CATS

A minimum of eight healthy adult cats at least one year of age and of optimal body weight shall be required to start the test. Queens in gestation or lactation shall be excluded. All animals starting the test must pass an initial physical examination by a veterinarian. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. A minimum of 30 cats shall be required for developing a historical colony average, with data used to establish averages for all parameters coming from the same individual cats. A minimum of eight cats shall be required for the concurrent control group.

DIET

The same formulation shall be fed throughout the test although different production batches may be used. Diets fed to a concurrent control group or to cats in the determination of historical colony averages must have successfully passed a minimum feeding protocol for an adult maintenance claim for a cat food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall run for a minimum of 26 weeks and shall begin when cats are placed on the test diet.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Cats shall be fed *ad libitum* or based on energy needs. Fresh water shall be provided *ad libitum*. Any interruption in the feeding protocol must be disclosed and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1-~~A. Individual ~~D~~aily food consumption ~~may~~ shall be measured and recorded for all animals if any animal is removed for poor food intake.
- ~~2-~~B. Individual body weights shall be measured and recorded at the beginning, weekly, and at the end of the test.
- ~~3-~~C. Hemoglobin, packed cell volume, serum alkaline phosphatase, serum albumin and whole blood taurine shall be measured and recorded at the end of the test.
- ~~4-~~D. All cats shall be given a complete physical examination by a veterinarian at the beginning and at the end of the test. Each cat shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.
- ~~5-~~E. Any medication and the reason for its use must be recorded.
- ~~6-~~F. A number of cats, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Cats may be removed for poor food intake only during the first two weeks of the test. Data already collected from cats removed from the test shall be retained although it does not have to be included in the final results.
- ~~7-~~G. A necropsy shall be conducted on any cat which dies during the test and the findings recorded.

INTERPRETATION

- A. The diet shall fail if any cat shows clinical or pathological signs of nutritional deficiency or excess.
- B. All cats not removed for non-nutritional reasons or poor food intake must successfully finish the test.
- C. No individual cat shall lose more than 15% of its initial body weight. The average percent body weight change (final compared to initial) shall not be less than either:
 - ~~D-~~ 1- Negative ten percent; or
 - ~~+~~2- The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.05$, if $n=8$ per group).
- ~~E-~~D- The average final hemoglobin, packed cell volume, whole blood taurine and serum albumin values shall not be less than either:
 1. a- Hemoglobin - 10.0 g/dl (no individual < 8.0 g/dl);
~~a-~~b- PCV - 30% (no individual $< 24\%$);
~~b-~~c- Taurine - 300 nmole/ml (no individual < 200 nmole/ml);
~~c-~~d- Albumin - 2.8 g/dl (no individual < 2.4 g/dl); or
 2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).
- ~~F-~~E- The average final serum alkaline phosphatase value shall not be greater than either:
 1. 100 IU/L (no individual > 200 IU/L); or

2. The historical colony average plus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
3. The average for the concurrent control group, plus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).

OPTIONAL PROCEDURES

The testing requirements for a maintenance cat food may be met by successfully performing either the growth or gestation/lactation protocols in lieu of performing the maintenance protocol.

MINIMUM FEEDING PROTOCOL FOR PROVING A GROWTH CLAIM FOR A DOG FOOD

PUPPIES

A minimum of eight puppies from three different litters shall be required to start the test. The puppies shall be no older than eight weeks of age and weaned. All puppies starting the test must pass an initial physical examination by a veterinarian. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. Historical colony averages for weight gain of puppies must be developed for each sex. Colony statistics shall be calculated with at least 30 males to determine the colony male average weight gain ($\mu_{\text{MaleColony}} \pm \text{standard deviation } (\sigma_{\text{MaleColony}})$. In addition, at least 30 females shall be used to determine the colony female average weight gain ($\mu_{\text{FemaleColony}} \pm \text{standard deviation } (\sigma_{\text{FemaleColony}})$. An equal sex distribution is recommended for all groups. If equal sex distribution is not feasible, appropriate corrections must be made. A minimum of 30 puppies shall be required for developing the historical colony averages for parameters other than weight gain with all data used to establish averages for all parameters coming from the same individual puppies. When using a concurrent control group, a minimum of eight puppies for the control group and eight puppies for the test group derived from at least three different litters (same sex distribution as the test group) shall be required to form the test and for the concurrent control groups. The test group shall have the same gender distribution as the concurrent control group. Breed distribution shall be similar in all groups.

DIET

The same formulation shall be fed throughout the test, although different production batches may be used. Diets fed to a concurrent control group or to puppies in the determination of historical colony averages must have successfully passed a minimum feeding protocol for a growth claim for a dog food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall run for a minimum of 10 weeks.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Puppies shall be fed *ad libitum* or based on energy needs. Fresh water shall be provided *ad libitum*. Puppies may be fed individually or in groups. The historical or concurrent control groups shall be fed in a manner similar to that of the treatment group. Any interruption in the feeding protocol must be disclosed and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1-A.~~ Individual ~~D~~daily food consumption ~~may~~ shall be measured and recorded for all animals if any animal is removed for poor food intake.
- ~~2-B.~~ Individual body weights shall be measured and recorded at the beginning, weekly, and at the end of the test.
- ~~3-C.~~ Hemoglobin, packed cell volume, and serum albumin shall be measured and recorded at the end of the test.

- 4-~~D.~~ All puppies shall be given a complete physical examination by a veterinarian at the beginning and at the end of the test. Each puppy shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.
- 5-~~E.~~ Any medication and the reason for its use must be recorded.
- 6-~~F.~~ A number of puppies, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Puppies may be removed for poor food intake only during the first two weeks of the test. Data already collected from puppies removed from the test shall be retained although it does not have to be included in the final results.
- 7-~~G.~~ A necropsy shall be conducted on any puppy which dies during the test and the findings recorded.

INTERPRETATION

- A. The diet shall fail if any puppy shows clinical or pathological signs of nutritional deficiency or excess.
- B. All puppies not removed for non-nutritional reasons or poor food intake must successfully finish the test.
- C. The average body weight gain shall not be less than either:
1. ~~75%-80%~~ of the historical colony average, with averages for males and females determined separately for both the test and colony groups; or
 2. ~~The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals.~~ The average body weight gain for N puppies (N ≥ 8) shall not be less than either the adjusted historical colony average minus 1.64 times the standard error. (See APPENDIX 1 for mathematical formulas required to calculate adjusted historical colony average and standard error regarding weight gain);
or
 3. ~~The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at p<0.01, if n=8 per group).~~ The average body weight gain shall not be less than the average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at p<0.05, if N=8 per group). (See APPENDIX 2 for mathematical formulas required to calculate the standard error of the difference of the two group averages for weight gain).
- D. The average final hemoglobin, packed cell volume and serum albumin values shall not be less than either:
1. a. Hemoglobin - 11.0 g/dl (no individual <9.0 g/dl),
b. PCV - 33% (no individual <27%),
c. Albumin - 2.6 g/dl (no individual <2.2 g/dl); or
 2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at p≤0.01, if n=8 per group).

MINIMUM FEEDING PROTOCOL FOR PROVING A GROWTH CLAIM FOR A CAT FOOD

KITTENS

A minimum of eight kittens from three different queens shall be required to start the test. The kittens shall be no older than nine weeks of age and weaned. All kittens starting the test must pass an initial physical examination by a veterinarian. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. Historical colony averages for weight gain of kittens must be developed for each sex. Colony statistics shall be calculated with at least 30 males to determine the colony male average weight gain ($\mu_{\text{MaleColony}} \pm \text{standard deviation } (\sigma_{\text{MaleColony}})$. In addition, at least 30 females shall be used to determine the colony female average weight gain ($\mu_{\text{FemaleColony}} \pm \text{standard deviation } (\sigma_{\text{FemaleColony}})$. ~~An equal sex~~

~~distribution is recommended for all groups. If equal sex distribution is not feasible, appropriate corrections must be made. A minimum of 30 kittens shall be required for developing the historical colony averages for parameters other than weight gain with all data used to establish averages for all parameters coming from the same individual kittens. When using a concurrent control group, a minimum of eight kittens for the control group and eight kittens for the test group derived from at least three different queens (same sex distribution as the test group) shall be required to form the test and for the concurrent control groups. The test group shall have the same gender distribution as the concurrent control group. Breed distribution shall be similar in all groups.~~

DIET

The same formulation shall be fed throughout the test, although different production batches may be used. Diets fed to a concurrent control group or to kittens in the determination of historical colony averages must have successfully passed a minimum feeding protocol for a growth claim for a cat food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall run for a minimum of 10 weeks.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Kittens shall be fed *ad libitum* or according to energy needs. Fresh water shall be provided *ad libitum*. Kittens may be fed individually or in groups. The historical or concurrent control groups shall be fed in a manner similar to that of the treatment group. Any interruption in the feeding protocol must be disclosed and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1.-A. Individual Daily food consumption may shall be measured and recorded for all animals if any animal is removed for poor food intake.~~
- ~~2.-B. Individual body weights shall be measured and recorded at the beginning, weekly, and at the end of the test.~~
- ~~3.-C. Hemoglobin, packed cell volume, whole blood taurine, and serum albumin shall be measured and recorded at the end of the test.~~
- ~~4.-D. All kittens shall be given a complete physical examination by a veterinarian at the beginning and at the end of the test. Each kitten shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.~~
- ~~5.-E. Any medication and the reason for its use must be recorded.~~
- ~~6.-F. A number of kittens, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Kittens may be removed for poor food intake only during the first two weeks of the test. Data already collected from kittens removed from the test shall be retained although it does not have to be included in the final results.~~
- ~~7.-G. A necropsy shall be conducted on any kitten which dies during the test and the findings recorded.~~

INTERPRETATION

- A. The diet shall fail if any kitten shows clinical or pathological signs of nutritional deficiency or excess.
- B. All kittens not removed for non-nutritional reasons or poor food intake must successfully finish the test.
- C. The average body weight gain shall not be less than either:
 1. 80% of the historical colony average, with averages for males and females determined separately for both the test and colony groups; or
 2. ~~The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals. The average body weight gain for N kittens (N ≥ 8) shall not be less than either the adjusted historical colony average minus 1.64 times the standard error. (See APPENDIX 1 for mathematical formulas required to calculate adjusted historical colony average and standard error regarding weight gain);~~
or
 3. ~~The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of~~

the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group)
The average body weight gain shall not be less than the average for the concurrent control group,
minus the allowance for normal variation. The allowance for normal variation is defined as 1.76
times the pooled estimate of the standard error of the difference of the two group averages (one-
tailed, two sample t-test at $p \leq 0.05$, if $N=8$ per group). (See APPENDIX 2 for mathematical
formulas required to calculate the standard error of the difference of the two group averages for
weight gain).

- D. The average final hemoglobin, packed cell volume, whole blood taurine and serum albumin values shall not be less than either:
1. a. Hemoglobin - 10.0 g/dl (no individual < 8.0 g/dl),
b. PCV - 29% (no individual $< 26\%$),
c. Taurine - 300 nmole/ml (no individual < 200 nmole/ml),
d. Albumin - 2.7 g/dl (no individual < 2.4 g/dl); or
 2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).

MINIMUM FEEDING PROTOCOL FOR PROVING A GESTATION/LACTATION CLAIM FOR A DOG FOOD

DOGS

Enough bitches shall be used to ensure that a minimum of eight pregnant bitches start the test. The bitches must be in at least their second heat period and at least one year of age. All bitches starting the test must pass an initial physical examination by a veterinarian. There is no specific size or breed requirement, but the bitches and studs must be of the same breed. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. A minimum of 30 bitches shall be required for developing a historical colony average, with data used to establish averages for all parameters coming from the same individual bitches. A minimum of eight bitches shall be required for the concurrent control group. Breed distribution must be similar in all groups.

~~For larger litters, puppies may be removed to normalize litter size to the following levels:~~

<u>Adult Dog Weights</u>	<u>Number in Litter</u>
<u>Less than 20#</u>	<u>5</u>
<u>20-50#</u>	<u>6</u>
<u>Greater than 50#</u>	<u>8</u>

~~Removed puppies may be transferred to bitches of the same breed with smaller litters.~~

DIET

The same formulation shall be fed throughout the test, although different production batches may be used. Diets fed to a concurrent control group or to bitches in the determination of historical colony averages must have successfully passed the minimum feeding protocol for a gestation/lactation claim for a dog food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall begin at or before estrus, and shall end when the puppies are 4 weeks of age, independent of age at weaning.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Animals shall be fed *ad libitum* or based on energy needs which are affected by the size of litter being nursed. Fresh water shall be provided *ad libitum*. Any interruption in the feeding protocol must be disclosed, and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1-A.~~ Individual ~~D~~ daily food consumption for the bitch during gestation and for the bitch and her puppies during lactation ~~may shall~~ be measured and recorded for all animals if any animal is removed for poor food intake.
- ~~2-B.~~ For each bitch, body weights shall be measured and recorded at breeding, weekly during gestation, within 24 hours after whelping, weekly during lactation, and at the end of the test. For the puppies, body weights shall be measured and recorded within 24 hours after birth, weekly, and at the end of the test.
- ~~3-C.~~ The litter size at birth, at one day of age, and at the end of the test shall be recorded. Stillbirths and congenital abnormalities shall be recorded.
- ~~4-D.~~ Hemoglobin, packed cell volume, and serum albumin shall be measured and recorded for the bitch at the end of the test.
- ~~5-E.~~ All bitches shall be given a complete physical examination by a veterinarian at the beginning of the test, and at the end of the test. Each bitch shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded. All puppies shall be given a complete physical examination by a veterinarian within 72 hours after birth, and at the end of the test. Each puppy shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.
- ~~6-F.~~ Any medication and the reason for its use must be recorded.
- ~~7-G.~~ A number of bitches, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Bitches may be removed for poor food intake only during the first two weeks of the test. Data already collected from bitches or puppies removed from the test shall be retained although it does not have to be included in the final results.
- ~~8-H.~~ A necropsy shall be conducted on any bitch or puppy which dies during the test and the findings recorded.

INTERPRETATION

- A. The diet shall fail if any bitch or puppy shows clinical or pathological signs of nutritional deficiency or excess.
- B. All bitches not removed for non-nutritional reasons or poor food intake must successfully finish the test. Eighty percent of all one-day-old puppies must survive and successfully finish the test.
- C. The pregnant bitches on the test shall show weight gain during gestation. The average percent body weight change (breeding through the end of the test) of the bitches shall not be less than either:
 1. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 2. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$ 0.05, if $n=8$ per group).
- D. The average weight of the puppies at the end of the test shall not be less than either:
 1. ~~75%~~80% of the historical colony average; or
 2. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals. (See APPENDIX 1 for mathematical formulas required to calculate adjusted historical colony average and standard error regarding weight gain); or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$ 0.05, if $n=8$ per group). (See APPENDIX 2 for mathematical formulas required to calculate the standard error of the difference of the two group averages for weight gain).
- E. At the end of the test, the average litter size of the bitches completing the test shall not be less than either:
 1. 80% of the historical colony average; or
 2. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or

3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at ~~$p \leq 0.04$~~ $p \leq 0.05$, if $n=8$ per group).
- F. The average final hemoglobin, packed cell volume, and serum albumin values shall not be less than either:
1. a. Hemoglobin - 10.0 g/dl (no individual <8.0 g/dl),
b. PCV - 30% (no individual $<24\%$),
c. Albumin - 2.4 g/dl (no individual <2.2 g/dl); or
 2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).

MINIMUM FEEDING PROTOCOL FOR PROVING A GESTATION/LACTATION CLAIM FOR A CAT FOOD

CATS

Enough queens shall be used to ensure that a minimum of eight pregnant queens start the test. The queens must be in at least their second heat period and at least one year of age. All queens starting the test must pass an initial physical examination by a veterinarian. Historical colony averages shall be acquired from a similar population of animals within the same testing facility that accurately represents the size and breed of the test group. A minimum of 30 queens shall be required for developing a historical colony average, with data used to establish averages for all parameters coming from the same individual queens. A minimum of eight queens shall be required for the concurrent control group.

~~For litters larger than 5 kittens, the additional kittens may be removed. Removed kittens may be transferred to queens with smaller litters.~~

DIET

The same formulation shall be fed throughout the test, although different production batches may be used. Diets fed to a concurrent control group or to queens in the determination of historical colony averages must have successfully passed the minimum feeding protocol for a gestation/lactation claim for a cat food. It may be helpful to consider diet type (i.e., dry vs. semi-moist vs. canned) in establishing colony averages.

DURATION OF TEST

The test shall begin at or before estrus, and shall end when the kittens are 6 weeks of age, independent of age at weaning.

FEEDING PARAMETERS

The test diet shall be the sole source of nutrients except for water. Animals shall be fed *ad libitum* or based on energy needs which are affected by the size of litter being nursed. Fresh water shall be provided *ad libitum*. Any interruption in the feeding protocol must be disclosed, and may invalidate the test.

CLINICAL OBSERVATIONS AND MEASUREMENTS

- ~~1-A.~~ Individual Daily food consumption for the queen during gestation and for the queen and her kittens during lactation ~~may~~shall be measured and recorded for all animals if any animal is removed for poor food intake.
- ~~2-B.~~ For each queen, body weights shall be measured and recorded at breeding, weekly during gestation, within 24 hours after queening, weekly during lactation, and at the end of the test. For the kittens, body weights shall be measured and recorded within 24 hours after birth, weekly, and at the end of the test.
- ~~3-C.~~ The litter size at birth, at one day of age, and at the end of the test shall be recorded. Stillbirths and congenital abnormalities shall be recorded.
- ~~4-D.~~ Hemoglobin, packed cell volume, whole blood taurine, and serum albumin shall be measured for the queen at the end of the test.

- ~~5-E.~~ All queens shall be given a complete physical examination by a veterinarian at the beginning of the test and at the end of the test. Each queen shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded. All kittens shall be given a complete physical examination by a veterinarian within 72 hours after birth, and at the end of the test. Each kitten shall be evaluated as to general health, body and hair coat condition, and comments shall be recorded.
- ~~6-F.~~ Any medication and the reason for its use must be recorded.
- ~~7-G.~~ A number of queens, not to exceed 25% of those starting the test, may be removed for non-nutritional reasons or poor food intake. The reason for their removal must be recorded. Queens may be removed for poor food intake only during the first two weeks of the test. Data already collected from queens or kittens removed from the test shall be retained although it does not have to be included in the final results.
- ~~8-H.~~ A necropsy shall be conducted on any queen or kitten which dies during the test and findings recorded.

INTERPRETATION

- A. The diet shall fail if any queen or kitten shows clinical or pathological signs of nutritional deficiency or excess.
- B. All queens not removed for non-nutritional reasons or poor food intake must successfully finish the test. Eighty percent of all one-day-old kittens must survive and successfully finish the test.
- C. The pregnant queens on the test shall show weight gain during gestation. The average percent body weight change (breeding through the end of the test) of the queens shall not be less than either:
1. Negative ten percent (no individual <-15%); or
 2. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at ~~p<0.04~~0.05, if n=8 per group).
- D. The average weight of the kittens at the end of the test shall not be less than either:
1. 80% of the historical colony average; or
 2. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root number of test animals. (See APPENDIX 1 for mathematical formulas required to calculate adjusted historical colony average and standard error regarding weight gain); or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at ~~p<0.04~~0.05, if n=8 per group). (See APPENDIX 2 for mathematical formulas required to calculate the standard error of the difference of the two group averages for weight gain).
- E. At the end of the test, the average litter size of the queens completing the test shall not be less than either:
1. 80% of the historical colony average; or
 2. The historical colony average minus ~~2.33~~1.64 times the standard error. The standard error is defined as the colony standard deviation divided by the square root number of test animals; or
 3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as ~~2.62~~1.76 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at ~~p<0.04~~0.05, if n=8 per group).
- F. The average final hemoglobin, packed cell volume, whole blood taurine and serum albumin values shall not be less than either:
1. a. Hemoglobin - 9.5 g/dl (no individual <8.0 g/dl),
b. PCV - 29% (no individual <26%),
c. Taurine - 300 nmole/ml (no individual <200 nmole/ml),
d. Albumin - 2.7 g/dl (no individual <2.4 g/dl); or

2. The historical colony average minus 2.33 times the standard error. The standard error is defined as the colony standard deviation divided by the square root of the number of test animals; or
3. The average for the concurrent control group, minus the allowance for normal variation. The allowance for normal variation is defined as 2.62 times the pooled estimate of the standard error of the difference of the two group averages (one-tailed, two sample t-test at $p \leq 0.01$, if $n=8$ per group).

CRITERIA FOR SUBSTANTIATION OF ~~CONTINUED VALIDITY OF NUTRITIONAL~~ ADEQUACY BASED ON FEEDING PROTOCOL RESULTS

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DEFINITION

A protocol substantiated formula is a dog or cat food, whether marketed or not, that has passed one or more AAFCO dog or cat food feeding protocols for substantiation of nutritional adequacy of one or more life stages. These are products that comply with Model Regulation (MR) PF7(a)(2) or PF7(b)(2)(B). A protocol substantiated formula may be a Product Family lead product. A protocol substantiated formula may carry a claim of nutritional adequacy substantiated via animal feeding protocol in compliance with PF7(c)(1)(B). A Product Family Member can be established according to the Procedures For Establishing Product Families. These Product Family Members may also carry a claim of nutritional adequacy substantiated via animal feeding protocol in compliance with PF7(c)(1)(B), provided they have been tested in a feeding protocol for determining metabolizable energy.

FORMULA CHANGES

Substitution of one or more different ingredients for ingredients used in a protocol substantiated formula necessitates redoing the previously passed protocol(s) in order to retain a nutritional adequacy claim permitted by MR PF7(c)(1)(B), unless the manufacturer has data on file that are sufficient to establish that the protocol substantiated formula is the lead member of a Pet Food Product Family and the new formula meets the criteria for being a member of the product family for bearing the claim specified in MR PF7(c)(1)(B). Substitution of one or more different ingredients for ingredients used in a Product Family Member formula necessitates re-establishing that the new formula meets the criteria for being a member of the product family.

A change in ingredient proportions in a protocol substantiated formula that mandates a reordering of the listed ingredients according to their predominance of weight necessitates redoing the previously passed protocol(s) in order to retain a nutritional adequacy claim permitted by MR PF7(c)(1)(B), unless the manufacturer has data on file that are sufficient to establish that the protocol substantiated formula is the lead member of a Pet Food Product Family and the new formula meets the criteria for being a member of the product family.

MARKETED FORMULAS

Every 5 years a manufacturer shall demonstrate ~~continued validity~~ of its marketed protocol substantiated formula products that bear a nutritional adequacy statement permitted in MR PF7(c)(1)(B) by producing data sufficient to show that the mean of six analyses from six independent, randomly selected batches of the currently marketed formula contains at least 95% of each of the key nutrients (dog and cat foods: crude protein, calcium, phosphorus, zinc, lysine, thiamine; plus for cat foods: potassium and taurine) used for establishment of a family member product using data from analyses of the original formula used in the feeding protocol(s) as the original nutrient values. The absence of data necessary to establish the original protocol substantiated formula as a lead product in a Pet Food Product Family for formulas marketed for 5 or more years necessitates redoing the previously passed protocol(s) in order to retain a nutritional adequacy claim permitted by MR PF7(c)(1)(B).

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Every 5 years, a manufacturer shall demonstrate ~~continued validity~~ of its Product Family Members that bear a nutritional adequacy statement permitted in MR PF7(c)(1)(B) by producing data sufficient to show that the currently marketed formula meets the criteria for being a member of a Pet Food Product Family

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using data from the original feeding protocol(s) as the lead product criteria for establishment of a Pet Food Product Family.

APPENDIX 1 CALCULATION FORMULAS FOR ADJUSTED COLONY AVERAGE AND STANDARD ERROR FOR WEIGHT GAIN OF PUPPIES OR KITTENS.

These mathematical formulas are to be used when evaluating weight gain produced by a test diet against the historical colony average.

$$\text{Adjusted Colony Average} = \frac{(\#TestMales \times \mu_{MaleColony}) + (\#TestFemales \times \mu_{FemaleColony})}{N}$$

The Colony standard error is defined as:

$$SE = \sqrt{\frac{\sigma_{MaleColony}^2 + \sigma_{FemaleColony}^2}{2 \times N}} ; \text{ or}$$

APPENDIX 2 CALCULATION FORMULAS FOR STANDARD ERROR OF THE DIFFERENCE BETWEEN TEST AND CONCURRENT CONTROL GROUP FOR WEIGHT GAIN OF PUPPIES OR KITTENS

These mathematical formulas are to be used when evaluating weight gain produced by a test diet against weight gain produced by a concurrent control group fed a diet having successfully passed the same feeding protocol.

Calculate the following test statistics:

<u>Puppies/Kittens</u>	<u>Standard Deviation</u>	<u>n</u>
Male Test (MT)	$\frac{SD_{MT}}$	$\frac{n_{MT}}$
Female Test (FT)	$\frac{SD_{FT}}$	$\frac{n_{FT}}$
Male Control (MC)	$\frac{SD_{MC}}$	$\frac{n_{MC}}$
Female Control (FC)	$\frac{SD_{FC}}$	$\frac{n_{FC}}$

$$SE_{diff.} = \sqrt{\left[\frac{(n_{MT} - 1) \times SD_{MT}^2 + (n_{MC} - 1) \times SD_{MC}^2 + (n_{FT} - 1) \times SD_{FT}^2 + (n_{FC} - 1) \times SD_{FC}^2}{n_{MT} + n_{MC} + n_{FT} + n_{FC} - 4} \right] \times \frac{2}{N}}$$

Note: The test and control groups of N each (N ≥ 8) must contain at least 2 male pairs or at least 2 female pairs.

If the test (T) and control (C) are composed of all male or all female offspring then the standard error of the difference of the two group averages simplifies to:

$$SE_{diff.} = \sqrt{\frac{SD_T^2 + SD_C^2}{N}}$$