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Scrapie infections initiated at varying doses: an analysis of 117 titration experiments

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An analysis of 117 titration experiments in the murine scrapie model is presented. The experiments encompass 30 years' work and a wide range of experimental conditions. To check that the experimental designs were reasonably consistent over time, comparisons were made of size, duration, source of inoculum, etc., in each experiment. These comparisons revealed no systematic trends that would render invalid comparisons across experiments. For 114 of the experiments it was possible to calculate the dose at which half of the challenged animals were infected (the ID_{50}). These 114 experiments were then combined on the basis of relative dose (i.e. tenfold dilution relative to the ID_{50}). This created a data set in which over 4000 animals were challenged with doses of scrapie ranging from four orders of magnitude below to five orders of magnitude above the ID_{50}. Analysis of this data reveals that mean incubation periods rise linearly with logarithmic decreases in dose. A one unit increase in relative dose (i.e. a tenfold increase in actual dose) will, on average, decrease the incubation period by 25 days. At ID_{50} the average incubation period in this data set is 300 days. Within a single dose, in a single experimental model, incubation periods have a distribution close to normal. Variability in incubation period also rises linearly as dose decreases. There is no age or sex effect upon the probability of infection, but female mice have incubation periods that are, on average, nine days shorter than their male counterparts and young mice have incubation periods that are longer by seven days. Although many of these patterns are apparent in the results of single titration curves, they can be more rigorously investigated by considering the outcome for thousands of mice.

Keywords: transmissible spongiform encephalopathy; titration curve; incubation period; meta-analysis; scrapie; BSE

1. INTRODUCTION

Many British people have probably been exposed to the agent that causes bovine spongiform encephalopathy (BSE) and variant Creutzfeldt–Jakob disease (vCJD) (Collinge *et al*. 1996; Bruce *et al*. 1997; Scott *et al*. 1999); we hope at low dose. Whether low-dose exposure will lead to infection and, if so, with what natural history such infections will unfold are therefore questions of great interest. Three types of question stand out. First, is there a dose below which the probability of infection is zero? Second, once initiated how does the incubation period (both mean and distribution) vary as a function of the initiating dose? Current attempts to predict the future course of any vCJD epidemic are severely curtailed by lack of knowledge about even the shape of the incubation period distribution (Cousens *et al*. 1997; Ghani *et al*. 1998). Third, does age and/or sex affect the probability of acquiring infection and any subsequent incubation period? The current epidemiology of vCJD suggests that they may (Will *et al*. 1999).

To study questions about the outcome of infections initiated at varying doses requires the challenge of large numbers of animals—particularly so when interest lies in doses that are several orders of magnitude smaller than a dose that would infect half the animals challenged (the ID_{50}). Over the past 35 years very large numbers of titration experiments in the murine scrapie model have been carried out at the Neuropathogenesis Unit (NPU) in Edinburgh (see, for example, Dickinson *et al*. 1975; Fraser & Dickinson 1970, 1978; Dickinson & Fraser 1969; Kimberlin & Walker 1988; Bruce *et al*. 1991). These experiments were performed with many different combinations of mouse breeds, scrapie strains, routes of inoculation, etc., and they cannot therefore be directly compared on the basis of incubation period. However, for each different titration experiment the dose at which approximately half the animals were infected can be calculated and used to align the results for each experiment, enabling the analysis of these many different experiments as a coherent whole. This allows the creation of a database, which records the outcome of the challenge of many thousands of mice. These mice have been challenged with doses that range from four orders of magnitude below to five orders of magnitude above the ID_{50}.

2. MATERIAL AND METHODS

(a) *Database construction*

For each of the 4278 mice the information listed in table 1 was entered into a spreadsheet. These mice represent the results of

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117 different titration experiments. Within each individual titration experiment the first five factors of table 1 are constant. From the information in table 1 each animal was assigned one of five possible outcomes (intercurrent death, mistaken cull, survivor, scrapie with incubation period, scrapie without incubation period) following the rules described in table 2. Animals that suffered an intercurrent death or mistaken cull were excluded from analysis. The duration of the observation period (i.e. time from inoculation to death) and age at inoculation were calculated for each animal. In calculations concerning the duration of the incubation period, only those animals with known scrapie incubation period were considered.

(b) *Database properties and consistency*

The general properties of this database are summarized in figure 1 and table 3. Most experiments were initiated in the early 1970s. The assembly of this information is ongoing and more recent data are currently being collated. The 117 experiments use ten different mouse breeds and seven different scrapie strains (table 3). The majority of experiments were initiated using intracranial inoculation (figure 1*a*) and the source of infectious material was usually brain tissue, but occasionally spleen (data not shown). Comparison across 30 years of experiments requires a certain degree of consistency in the experimental conditions. Figure 1 shows that although there is variability in the size (figure 1*b*) and duration (figure 1*c*) of these experiments, there are no systematic trends that would render such comparisons invalid. It is worth noting that in many of these experiments a substantial number of animals (often 30% or more) die from other causes before the end of the experiment (figure 1*d*). The proportion that eventually develop scrapie varies widely across experiments, with no temporal trend (figure 1*e*). Finally, there was a cluster of five experiments using aged animals in the early 1970s, otherwise, the age of the animals at challenge has not changed greatly over the 30 years of experimentation (figure 1*f*).

(c) *Calculation of ID_{50}s*

In everything that follows we analyse the data using the concept of the relative dose. Thus rather than being interested in whether an animal was inoculated with, say, 1 g of infectious tissue diluted 10^5-fold, we wish to know the size of the inoculum relative to that which results in 50% of animals being infected. Thus the ID_{50} is assigned the value of 0, with a dose that is tenfold larger than the ID_{50} being assigned relative dose 1, or a dose that is 100-fold lower being assigned a relative dose of -2. In order to assign each dilution a relative dose, we need to calculate the ID_{50} for each of the 117 individual titration experiments. This was done using a mixture of statistical curve fitting and common sense, as outlined in figure 2. The standard method for analysing titration curve data is to use binary logistic regression with either a logit or probit link function (Armitage & Berry 1971). This statistical procedure requires that there should be at least two doses for which the proportion of animals infected is neither 1 nor 0. For those titration experiments where this was the case ($n = 54$), such fits were made using the statistical package Minitab (Anonymous 1998). The ID_{50} was calculated from the parameters of the best fitting regression line and rounded down to the nearest whole number (figure 2*a*). This procedure was performed using both logit and probit transformations. As expected (Armitage & Berry 1971), the two methods gave consistent answers. Because the titrations studied here use tenfold dilutions it is often the case that there is only one dose at which more than none and less than all the animals were infected. In such cases ($n = 44$), that dose was assigned to be the ID_{50} (figure 2*b*). Furthermore, in some experiments the number of animals is so small that the fraction of animals infected jumps straight from zero to one. In these cases ($n = 16$) the last dose at which no animals were infected was assigned to be the ID_{50} (figure 2*c*).

Table 1. *Information recorded for each mouse*

experiment id	NPU designated name
mouse breed	breed of mouse challenged
scrapie strain	scrapie strain used for challenge
tissue	tissue used to derive challenge material
route	route of inoculation
animal number	individual animal identification within the experiment
experimental group	dilution of inoculum
sex	sex of the challenged animal
date of birth	date of birth of the challenged animal
date of injection	date of inoculation
killed or died	whether the animal was killed or died
date of death	date on which the animal died
clinical +/−	whether or not the animal exhibited clinical signs of scrapie at death. Can be + or −
pathology +/−	whether or not the animal showed signs of scrapie pathology at post-mortem. Can be +, or − or not recorded
incubation period or survival	number of days between inoculation and death

(d) *Excluded experiments*

There were three titration experiments that could not be analysed using these three rules. Raw data from these three experiments are shown in table 4, then excluded from further analysis. These are the only three experiments that have been excluded from this analysis. They were among the smaller experiments having only four or five animals in each group. Otherwise these three experiments show no special properties: (a) begun in 1974 using ME7 in 16 VM mice, (b) begun in 1973 using ME7 in 18 C3H mice, (c) begun in 1972 using ME7 in 19 C3H mice. There is nothing obvious about these three experiments to cause concern that their exclusion will bias the results.

3. RESULTS

(a) *Overall titration curve*

Once the ID_{50} for each experiment has been calculated, each mouse can be assigned a relative dose—simply the difference between the dilution at ID_{50} and the actual dilution of that animal's challenge. A titration curve of all animals is then easily constructed. The results for this overall titration curve will be presented elsewhere (McLean & Bostock 2000). In brief, we find no evidence of a threshold dose below which the probability of infection becomes vanishingly small.

(b) *Average and distribution of the incubation period at different doses*

It is well known that as dose decreases the incubation period for scrapie increases. Not only does the mean for a group of animals increase, but so does the variability

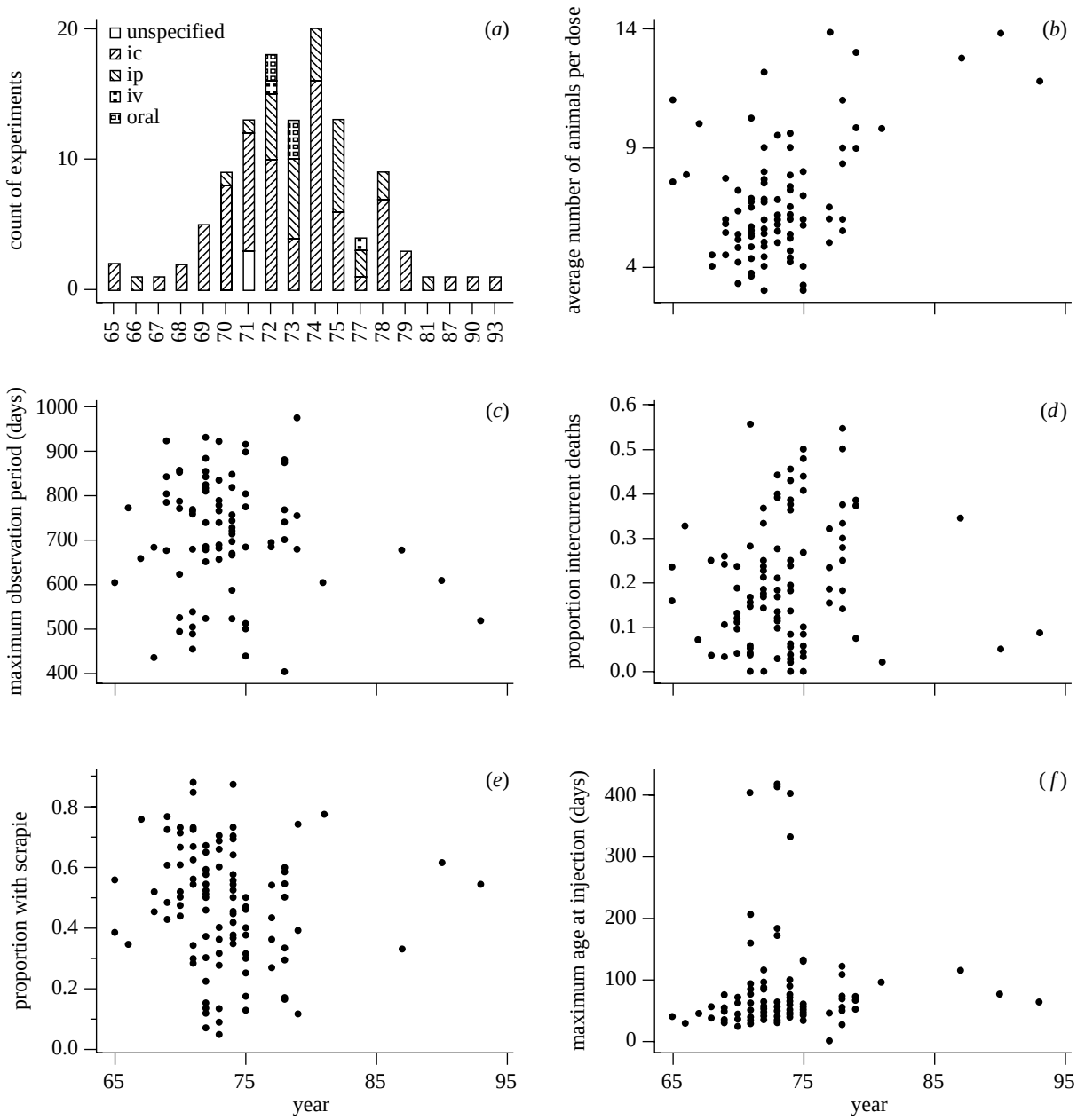

Figure 1. Database characteristics. The 117 titration experiments investigated here use a variety of experimental designs. However, there are no systematic shifts in the models used that would invalidate comparison across these 30 years of data. (*a*) The experiments analysed here were started between 1965 and 1993 and most were initiated using intracranial inoculation. Other aspects of the experimental design are also reasonably consistent through time: (*b*) the size of the experiments as represented by the average number of animals assigned to each dose; (*c*) the duration of the experiments as shown by the maximum period of observation; (*d*) survivorship of mice from extraneous causes of death as shown by the proportion of animals in each experiment succumbing to intercurrent death; (*e*) range of doses as reflected in the overall proportion infected; and, finally, (*f*) the age of the mice used.

Table 2. *Criteria for assigning individual animal outcome according to whether the animal was killed or died, did or did not exhibit clinical signs of scrapie and did or did not show scrapie pathology*

		pathology		
killed or died	clinical signs	+	−	not recorded
killed	+	scrapie with incubation period	mistaken cull	scrapie with incubation period
killed	−	scrapie without incubation period	survivor	survivor
died	+	scrapie with incubation period	intercurrent death	scrapie with incubation period
died	−	scrapie without incubation period	intercurrent death	intercurrent death

Table 3. *Distribution of experiments by mouse breed and scrapie strain combinations*

	scrapie strain							
mouse breed	115D	22A	22C	22L	79A	79V	ME7	total
BALB	—	—	—	—	1	—	3	4
BRVR	—	—	—	—	1	—	4	5
C3H	—	—	—	—	—	—	3	3
C57	1	5	6	3	4	—	16	35
CV	—	2	—	—	—	—	2	4
IM	—	2	—	—	—	—	—	2
RO	—	—	3	—	5	—	14	22
SM	—	—	—	—	2	—	1	3
VL	—	—	—	—	4	—	4	8
VM	—	22	1	—	—	1	7	31
total	1	31	10	3	17	1	54	117

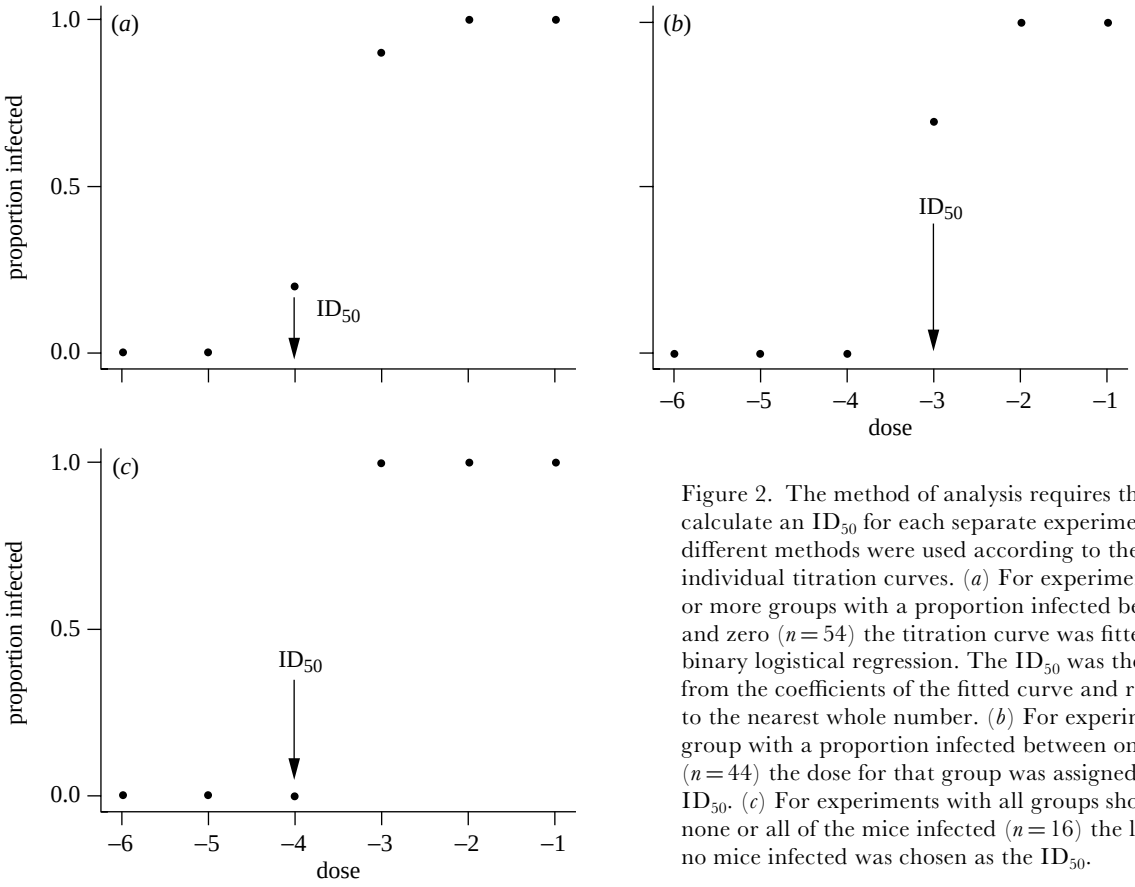

Figure 2. The method of analysis requires that we calculate an ID_{50} for each separate experiment. Three different methods were used according to the nature of the individual titration curves. (*a*) For experiments with two or more groups with a proportion infected between one and zero ($n = 54$) the titration curve was fitted using binary logistical regression. The ID_{50} was then calculated from the coefficients of the fitted curve and rounded down to the nearest whole number. (*b*) For experiments with one group with a proportion infected between one and zero ($n = 44$) the dose for that group was assigned to be the ID_{50}. (*c*) For experiments with all groups showing either none or all of the mice infected ($n = 16$) the last dose with no mice infected was chosen as the ID_{50}.

about that mean. This general trend is illustrated in figure 3 for three individual titration experiments with particularly large numbers of animals so that this pattern is well illustrated. While it is true that incubation periods for these murine scrapie models are remarkably tightly distributed at very high doses, the same is not true when doses are lower.

Having assigned relative doses to all infected animals, it is possible to look at patterns in incubation period distribution as a function of relative dose for all of the 1904 animals that became infected and for which the incubation period is known. These data are summarized in figure 4. Figure 4*a* shows a box-whisker plot of the incubation period for each of these mice as a function of relative dose. There seems to be a minimum incubation period of about 150 days. However, had any experiments using the mouse-passaged BSE strain 301V been included, this minimum would have been breached as this strain has an unusually short incubation period (Bruce *et al.* 1997). This minimum gives a substantial right skew to the incubation period distributions. The median incubation period falls linearly as dose increases, but levels off after three or four logs above ID_{50}. In these experiments the median incubation period at ID_{50} (relative dose = 0) is 300 days, for each tenfold increase in dose the incubation period decreases by about 25 days.

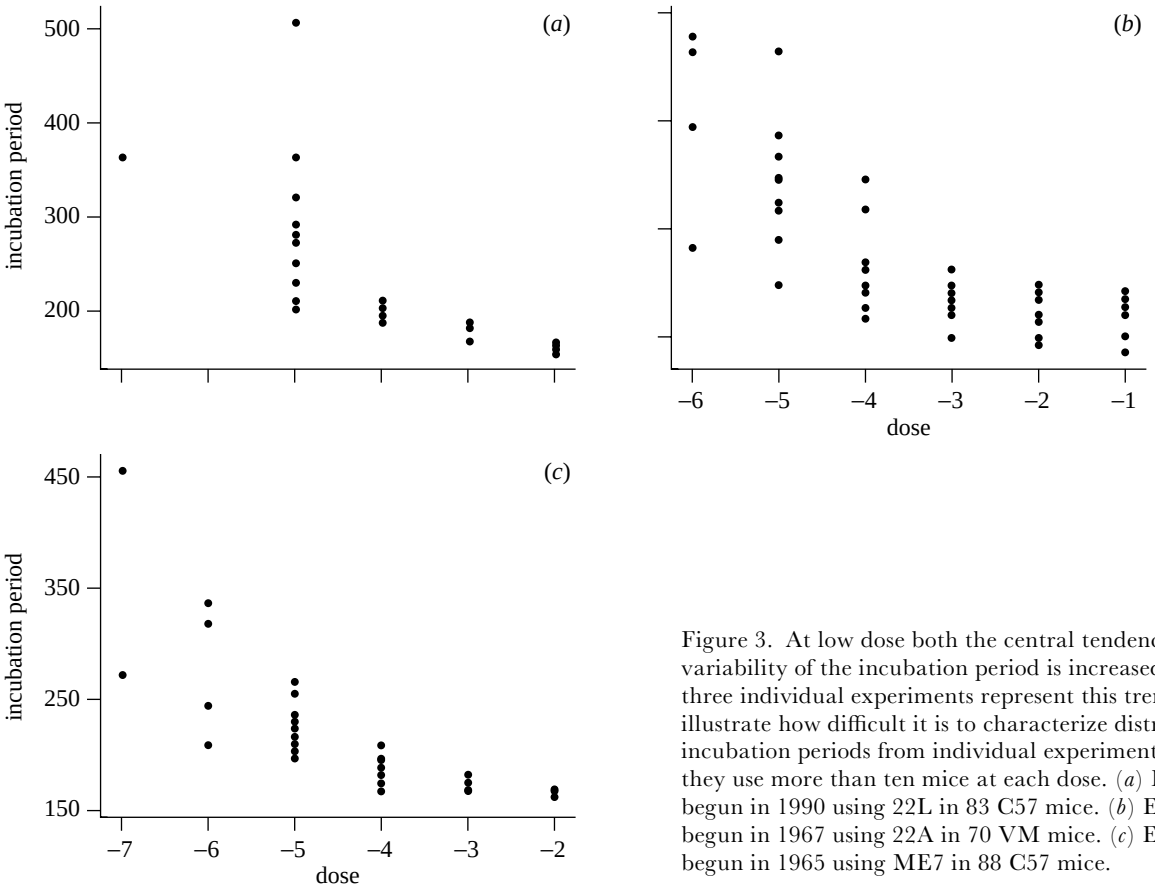

Figure 3. At low dose both the central tendency and variability of the incubation period is increased. These three individual experiments represent this trend, but illustrate how difficult it is to characterize distributions of incubation periods from individual experiments even when they use more than ten mice at each dose. (*a*) Experiment begun in 1990 using 22L in 83 C57 mice. (*b*) Experiment begun in 1967 using 22A in 70 VM mice. (*c*) Experiment begun in 1965 using ME7 in 88 C57 mice.

The shape of the incubation period distributions is shown in more detail in figure 4*b*, with a plot of the density distribution as a function of the incubation period. For clarity these are shown for just three relative doses. The same pattern is seen, the distribution moves towards higher incubation periods and becomes flatter and broader as the dose decreases. Variability also increases as dose decreases, as shown by this broadening of the density distribution in figure 4*b* and also by the increasing depth of the interquartile range boxes in figure 4*a*. However, presentation of the data in this manner includes variability across experiments. Since we are more interested in the variability within a single dose in a single experiment (but summarized across many experiments) we calculate for each mouse the difference between its incubation period and the average incubation period for the infected mice at that dose in that experiment. This is a more appropriate measure for the within-dose variability, but tells us nothing about shifts in central tendency across dose. If a group of mice has only one animal infected then this measure must automatically be zero, and such groups are more common at low doses, biasing this measure of within-group variability. In constructing figures 4*c* and 4*d* we therefore included only mice that came from a group where two or more animals became infected. Results are shown as a box-whisker plot in figure 4*c* and as a density histogram in figure 4*d*. The interquartile range for this within-group variability is just ten days at doses four logs above ID_{50}, and rises by about seven days for every tenfold decrease in dose. The detailed shape of the within-group distribution is shown for relative doses 0, 2 and 4 in figure 4*d*. A second method to remove some of the between-experiment variability is to consider just single combinations of a pair of mouse breed and scrapie strain. The two most common pairings (table 1) are ME7 in C57 mice (figure 4*e*) and 22A in VM mice (figure 4*f*). The patterns are conserved: both the median and the interquartile range of the incubation period decrease linearly with logarithmic increases in dose. However, the numerical details of change in median incubation period vary between the three plots in figures 4*a*, 4*e* and 4*f*. Thus while for all data combined a log increase in dose leads to a 25-day decrease in median incubation period, in C57 mice the decrease is 39 days and for 22A in VM mice the decrease is 27 days.

(c) *Age and sex as risk factors for infection*

We performed binary logistical regressions of the proportion infected as a function of relative dose. None of the factors strain, breed, route of inoculation or tissue has odds significantly different from one in such regressions. This is to be expected as these factors are always consistent across a single experiment and are therefore accounted for in the calculation of relative dose. Two further factors, age at challenge and sex, were also tested in a binary logistical regression against relative dose and neither was shown to have any significant impact on the probability that a mouse would become infected. Since each experiment contains animals of a mixture of ages and (usually) a mixture of sexes, this result does not follow from the use of relative dose as the covariate.

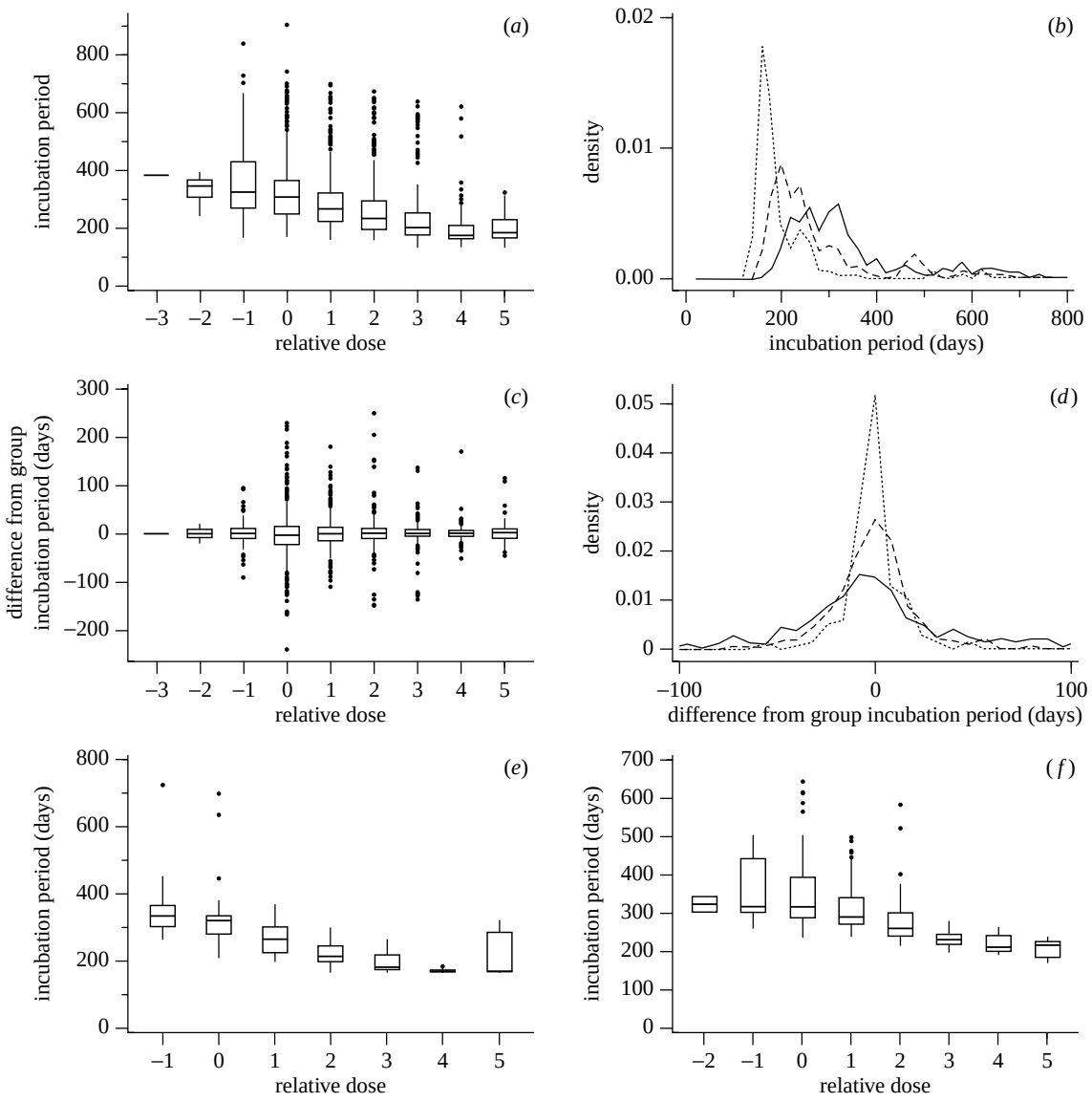

Figure 4. Trends in central tendency and variability of incubation periods at varying dose. (*a*) Box-whisker plot of incubation period as a function of relative dose (difference from ID_{50}). Relative dose is a logarithmic measure so a one unit increase represents a tenfold rise in the concentration of infectious material in the inoculum. As dose increases, median incubation period decreases in a linear fashion. (*b*) Detailed distribution of incubation periods at relative doses 4, 2 and 0. (*c*) Box-whisker plot of the difference of each mouse's incubation period from the average for its group (i.e. all infected mice at that dose in that experiment). Mice in groups where only one animal was infected are excluded. This treatment of the data removes between-experiment variability and is therefore a better method for investigating trends in the variability of incubation period with dose. Between the ID_{50} and four logs above, each tenfold increase in dose leads to a seven-day reduction in the interquartile range for the incubation period. (*d*) Detail of the distributions of difference from mean incubation period at relative doses 4, 2 and 0. These curves show that it would be reasonable to assume a normal distribution of the incubation period within an experimental group. (*e*) Box-whisker plot of incubation periods as a function of relative dose for the 16 experiments that used ME7 in C57 mice. (*f*) Box-whisker plot of incubation periods as a function of relative dose for the 22 experiments that used 22A in VM mice.

(d) *Age and sex effects on incubation period*

Although neither age nor sex was found to have any effect on the probability of infection, both have a small effect on incubation period (Bruce & Fraser 1981; Bruce & Dickinson 1985). In an analysis of variance of incubation period, with relative dose as a covariate and age at inoculation (above or below median) and sex as factors, all are significant. A one unit increase in relative dose (i.e. a tenfold increase in actual dose) will, on average, decrease the incubation period by 30 days. Female mice have incubation periods that are, on average, nine days shorter than their male counterparts (figure 5*a*) and young mice have incubation periods that are longer by seven days (figure 5*b*).

4. DISCUSSION

We introduced this paper with three questions. Does the probability of infection in murine scrapie models become zero at very low doses? How do the average and distribution of the incubation period change as functions of dose? Are there age and/or sex effects on infection

Table 4. *Raw data from three unusable experiments*

	experiment a		experiment b		experiment c	
dose	infected	tested	infected	tested	infected	tested
−6	3	4	—	—	—	—
−5	4	5	2	2	0	5
−4	2	4	3	6	0	6
−3	—	—	3	5	1	4
−2	2	3	2	5	0	3

probability or incubation period? By adopting the concept of relative dose (i.e. relative to ID_{50}) we have been able to standardize dosing across 114 different titration experiments and thus create a data set of sufficient size to begin to address these questions in a rigorous manner.

We found no evidence for a threshold dose below which the probability of infection became zero (McLean & Bostock 2000). The existence, or not, of such a threshold is of some practical interest in as much as these murine scrapie models inform about supposed oral exposure of humans to BSE. There are reasons, based on molecular biological work *in vitro* (Come *et al*. 1993; Wadsworth *et al*. 1999; Weissmann 1991), to believe that such a threshold may exist, and the subject has been the matter of substantial investigation using mathematical theory (Nowak *et al*. 1998; Payne & Krakauer 1998*a*,*b*). However, the effect is not observed in this data set.

We observe that the average incubation period increases linearly with logarithmic decrease in dose. This pattern would be consistent with a process of exponential growth of the pathogenic agent and disease onset at some threshold population size of the agent. This pattern has long been known (Dickinson *et al*. 1969; Eklund *et al*. 1964) and is sometimes used as a method for estimating dose from incubation period (Scott *et al*. 1997). However, variability in the incubation period also increases linearly as dose gets smaller, causing significant overlap in the incubation period across several doses—this is particularly so at lower doses.

We found no effect of age or sex on the probability of an animal becoming infected, but both factors impacted on the duration of the incubation period once an infection was initiated. Female mice and older mice have slightly shorter incubation periods. The current epidemiology of vCJD shows an excess of cases among young women. If such an effect were the result of incubation period differences, that would be consistent with our findings on the sex effect, but not the effect of age.

Although many of these patterns are apparent in the results of single titration curves, they can be more rigorously investigated by considering the outcome for thousands of mice. This is particularly so for the data on incubation period distributions as a function of dose. The method used here of combining data from many different titration experiments allows the assembly of such information from existing data archives without committing unreasonable resources to new experimentation.

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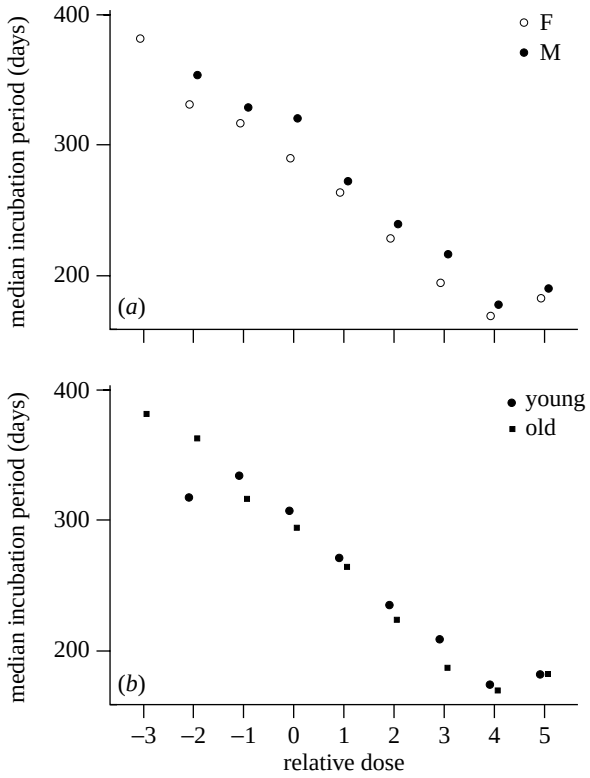

Figure 5. Sex and age effects on incubation period. (*a*) Female mice tend to have shorter incubation periods. (*b*) Young mice tend to have longer incubation periods. Here young mice are those that are at or below the median age of 36 days on the day of inoculation.

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