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Testing and analytical procedures for laboratory studies involving nonresponders during a limited observation period An illustration using male sexual behavior in rats

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Abstract

In many laboratory studies, a subpopulation of subjects fails to exhibit the response under investigation during the period of observation. For example, within any population of male rats, there is significant variation in the expression of sexual behavior in the presence of a receptive female. Some males may never display the full sequence of behaviors leading to ejaculation within the typical time frame of the testing session, with the resulting lack of behavioral response presenting problems in the analysis of the data. Conventional strategies range from screening such males from the study or dropping them from the analysis to constructing new variables based on estimates from existing parameters or increasing the length of the test session to capture sexual responses in a greater portion of males. Herein, we present an alternative strategy for analyzing data where outcomes are absent due to the limited observation period. Survival regression analysis enables inclusion of all subjects in the analysis whether or not they have shown the behavior of interest. Use of such a strategy not only has potential to reveal new results but also guards against bias from excluding nonresponders from the study or dropping more males from one experimental condition than another. Furthermore, this procedure can be helpful in generating the conditional probability (increase, decrease, or constant) of the response with the passage of time based on the hazard function and in estimating parameters for establishing an optimal behavioral test length for future studies.

Keywords: Male sexual behavior; Ejaculation; Intromission; Rat; Survival analysis; Survival regression analysis; Hazard function; 8-OH-DPAT; Serotonin

1. Introduction

1.1. Background

In many experimental studies, a subpopulation of subjects fails to exhibit the response under investigation during the period of observation. That is, some subjects may have a low probability of exhibiting the behavior (e.g., due to an experimental manipulation) or, alternatively, the period of observation may end before all subjects have had a chance to display the behavior. Given that some subjects fail to show the response, the researcher can categorize the behavior as either present or absent during the observation period and then compare overall rates of responding across experimental conditions. But, in viewing the response as an all-or-none phenomenon, the researcher ignores potentially valuable information — the fact that the occurrence of the behavior is actually distributed over time, with each subject demonstrating a specific latency to its onset. For some subjects, that latency may simply exceed the limits of the observation period, for others, it may approach infinity (e.g., in a true nonresponder). Such distributions, where the outcome of interest occurs at varying latencies but in which the outcome is absent in a portion of the cases due to termination of the observation period, are known as ''censored'' distributions and occur fairly frequently in laboratory investigations of behaviors. For example, studies investigating latencies to a variety of behaviors, including ingestive behavior (eating or drinking), maternal behavior (retrieval of pups), agonistic behavior (threat/attack), and sexual behavior (mounting,

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intromission, and ejaculation), often include cases that never display the relevant response within the allotted observation period.

Such studies call for an analytical strategy that enables use of the entire sample even though the outcome of interest occurs in only a portion of subjects. Specifically, survival analysis (Cox and Oates, 1984; Kalbfleisch and Prentice, 1980; Lawless, 1982; Miller, 1981) is ideal for distributions censored by termination of the observation period and is frequently used in longitudinal epidemiological studies where the outcome of interest is censored (e.g., disease survival rates). Although available for years, this statistical procedure has seldom been applied to experimental studies as those described above. Notable exceptions include reports by Bloch et al. (1993) and Liu et al. (1997) who have used the procedure in the study of male sexual behavior in rats. In its simplest nonparametric form, survival analysis enables the researcher to determine whether experimental groups show different ''survival functions,'' with that function being defined as the probability of displaying the relevant response.

In the present study, we used an example from the recent literature on male sexual behavior to illustrate the potential value of using survival *regression* analysis for the kinds of censored distributions typically encountered in experimental studies having limited observation periods. In contrast with nonparametric survival analysis (e.g., Kaplan Meier product limit estimator; Kalbfleisch and Prentice, 1980; Lawless, 1982), survival regression analysis imposes a specific structure on the data by making assumptions about the distribution of the dependent variable even though the distribution has been censored. By allowing the dependent variable to be treated as a random variable with a continuous distribution, the researcher is able to make inferences about the censored portion of the distribution based upon the sample of uncensored and censored observations. Specifically, a maximum likelihood estimation procedure can be used to draw conclusions about the hazard rate, that is, about the conditional probability of the behavior (increase, decrease, or constant) as a function of the passage of time. In addition, by using information from the censored part of the distribution, an optimal session length for capturing the majority of the responses can be estimated. Finally, explanatory (independent) variables can be incorporated into the model, with the resulting statistics interpreted as regression-like coefficients.

1.2. Example using male sexual behavior

Sexual behavior of the male rat toward a receptive female is characterized by a series of discrete mounts and/or intromissions, with each of the latter consisting of a single penile thrust into the vagina. This series typically terminates in ejaculation, although within a single testing session, the intromission–ejaculation sequence may be repeated one or more times. Whether the rat attempts and succeeds in the behavioral sequence leading to ejaculation depends on a variety of factors, including its prior sexual history, hormone status, the stimulus parameters, and other factors related to its motivational and/or arousal state (see Everitt, 1990; Pfaus, 1996, for review).

Studies assessing sexual behavior in the male rat typically rely on standard measures tied to the behavioral pattern described above, including the frequency of mounts, successful vaginal penetrations (intromissions), and ejaculations (Bitran and Hull, 1987; Pfaus, 1996). In addition, the latency to the onset of each of these behaviors is usually determined. Since rats may have multiple ejaculations within a single test session, this set of measures may be repeated, beginning with the latency to the onset of the first mount/intromission following the initial ejaculation (postejaculatory interval). Using such measures, researchers make inferences regarding constructs, such as ''sexual arousal,'' ''sexual motivation,'' and so on (Everitt, 1990; Pfaus, 1996).

In many studies, a portion of males may not exhibit the full sequence of behaviors delineated above. For example, within a 30-min testing session, some males may never begin mounting or intromitting, while others may intromit but never reach ejaculation. This situation presents a dilemma in analyzing studies focusing on the latency to ejaculation — a problem recently underscored in research investigating male sexual response to ejaculation-inducing agents (Ahlenius and Larsson, 1984; Haensel et al., 1991; Mos et al., 1990; Rowland and Houtsmuller, 1998). In that research, pharmacologically manipulated groups were compared on measures that included the number of ejaculations and, more importantly, the number of intromissions and the temporal latency to ejaculation. Yet, due to low motivation/arousal or to the limited observation period, the critical responses of intromitting or ejaculation were not displayed in a significant portion of the subjects, making it impossible to calculate ejaculation latencies for those males. Although simple analysis could compare the *rate* of ejaculation across groups (Rowland and Houtsmuller, 1998), the important question of whether the drug affected ejaculatory latency in a presumably representative sample presented a formidable challenge.

Accordingly, researchers have employed several strategies to deal with rats failing to intromit or ejaculate during the testing session. Such males are sometimes identified through pretesting and removed from the sample before the experiment begins (Smith et al., 1990), or they may be identified only after the study has been completed but then removed from the analysis or treated as if their data were missing (Rowland and Houtsmuller, 1998). These procedures, however, result in the loss of potentially valuable data and reduce the power of the analysis. Of greater concern, dropping these subjects may bias the distribution, particularly if a greater proportion of subjects is eliminated from one experimental condition than another.

A second strategy for dealing with nonejaculators is that of assigning ejaculatory latencies equal to the maximal length of the testing session (e.g., 30 min) or, less commonly, constructing a new variable to proxy for ejaculation

latency based on the interval from the onset of mounting to the end of the testing session (Arendash and Gorski, 1983; Commins and Yahr, 1984; De Jonge et al., 1989, 1990; Houtsmuller et al., 1994; Rowland and Houtsmuller, 1998). Both procedures are problematic. The former does not differentiate between rats that mount and intromit from those exhibiting no sexual response whatsoever — all are assigned latencies equal to the maximum time period even though they have shown different levels of sexual activity. The latter approach of calculating latency from the onset of mounts/intromission to the end of the testing session differentiates between nonejaculators that do and do not intromit. Yet, this strategy has the potential to generate artificially short latencies in subjects that do not initiate intromissions until the latter part of the testing session. Whereas short latencies generally suggest a high level of sexual responsivity, in these nonejaculating males they in fact represent fairly low levels of activity.

A third strategy is that of increasing the length of the testing session on the assumption that with more time, the proportion of males reaching ejaculation would increase. Indeed, studies investigating the effect of 8-OH-DPAT on ejaculatory latency have used testing sessions of varying lengths, anywhere from 15 to 60 min (Ahlenius and Larsson, 1984; Ahlenius et al., 1991; Fernandez-Guasti et al., 1992; Haensel et al., 1991, 1993; Mendelson and Gorzalka, 1986; Mos et al., 1990; Rowland and Houtsmuller, 1998; Smith et al., 1990). The lack of standardized test parameters not only makes comparisons across studies difficult but also raises the question of the optimal session length required to capture the majority of ejaculations and thus demonstrate an experimental effect.

1.3. Goals of this study

Previous studies on male sexual behavior have resorted to dropping cases and construction of problematic variables (as described above) to enable analysis where subjects fail to display the full sequence of sexual responses (i.e., censored data). However, the above problems can be readily addressed by using an analytical strategy applicable to situations where, because of characteristics of the subjects or a temporal constraint imposed by the length of the period of observation, responses are lacking for cases within the sample. Specifically, survival analysis enables use of the entire sample in the analysis even though the outcome (intromission and/or ejaculation) occurs in only a portion of subjects. As such, this analysis not only improves power by retaining the full sample but also guards against biases that might result from dropping cases for which critical outcomes are absent. Survival *regression* analysis (Greene, 2000), the parametric variation of conventional survival analysis and available in a number of software packages (Greene, 1998; SAS System, 1999), offers further advantages. This procedure can be used to generate estimates of parameters of the sexual behaviors for the entire sample (including the censored portion represented by the nonresponders). In doing so, it is possible to establish the conditional probability of a response with the passage of time and to determine an optimal test session length for assessing experimental effects.

2. Method

2.1. Description of the initial study and the problems encountered

Rowland and Houtsmuller (1998) recently published data on 106 castrated male Wistar rats, half of which were treated with 8-OH-DPAT (i.e., DPAT), a serotonergic agonist that purportedly decreases ejaculatory latency (Ahlenius et al., 1991; Fernandez-Guasti et al., 1992; Smith et al., 1990). In this study, the effects of three experimentally manipulated variables were assessed on ejaculatory latency: drug (DPAT) vs. saline treatment; the level of testosterone (below normal, about normal, and above normal); and the amount of prior mating experience (experienced vs. naive). Although the effect of DPAT on ejaculatory latency had been well established, our study attempted to resolve the controversy of whether normal levels of testosterone were essential to this effect (Haensel et al., 1993) and, further, whether sexual experience played a role (Mos et al., 1990). Since both testosterone and sexual experience sustain and/or increase the probability of the response (Damassa et al., 1977; Davidson, 1966; Larsson, 1978; Lopez et al., 1999), inexperienced males with low testosterone would have low arousal potential and therefore be less likely to intromit and/or ejaculate. Anticipating that a significant portion of these males might not display sexual activity within a typical 15-min test session, our study employed a testing session of 45 min, 15–30 min longer than many similar studies. Nevertheless, a high number of males in the low arousal conditions still failed to intromit and/or ejaculate, so for the analysis of ejaculatory latencies, we resorted to the conventional strategies mentioned above. Specifically, we had to drop over a third of the males from the analysis because they never began the sequence of intromissions. This not only resulted in a considerable loss of data but also a loss predominantly restricted to the low testosterone and inexperienced groups. Indeed, we essentially eliminated the low testosterone group ($n = 38$) from most analyses, preventing us from testing an effect related to that condition. For the remaining subjects showing intromissions but not ejaculations, we constructed latency variables based on the overall length of the testing session as described above. Here, too, we encountered a problem because preliminary analysis indicated that males treated with DPAT began intromitting later in the test session than nontreated males, the result of the well-known stereotypic motor response associated with DPAT treatment (Blackburn et al., 1984; Hillegaart et al., 1991). The systematic bias introduced by this difference made it impossible to use a latency variable based on the

interval between the onset of intromitting and the end of the test session. Thus, we were limited to using the overall length of the testing session as a measure of ejaculatory latency for nonejaculating males. Although conventional ANOVA indicated differences between drug- and non-drug-treated subjects as well as a drug by experience effect, the construction of this latency variable to compensate for the censored data, along with the elimination of so many subjects, was, in our view, far from satisfactory.

2.2. Reanalysis using the survival regression model

A more suitable analytical procedure for the data described above is that of survival regression analysis. With respect to our study, it enabled inclusion of all subjects in the analysis, whether or not they ejaculated, generated the conditional probability of the response over time based on the hazard function, and helped establish an optimal testing length for future studies.

For these analyses, the two random (dependent) variables of interest were the latency to ejaculation and the latency to the first mount or intromission. Mounts and intromissions were combined because they both reflect the clear onset of sexual activity in the male rat. The dependent variables were denoted as y and were censored at $y = 45$ min (the length of the test session). These random variables (y) can be described as having the probability distribution $f(y)$ and the cumulative distribution $F(y)$, the latter of which gives the probability of the random variable (in our case, either mounts/intromission or ejaculation) occurring by the time y. The survival function, defined as $S(y) = 1 - F(y)$, gives the probability that mounts/intromission or ejaculation will take at least time y to occur. Finally, the hazard function, $h(y) = f(y)/S(y)$, indicates the conditional probability of the event (mount/intromissions or ejaculation), given that the event has not occurred by time y.

To specify the survival model, we adopted a Weibull distribution. This distribution allows the hazard function to be increasing, constant, or decreasing in y. Specifically, the data determine whether the probability of mounts/intromissions increases or decreases the longer ''nonintromission'' persists, and likewise, whether the probability of ejaculation increases or decreases the longer ''nonejaculation'' persists. The explanatory variables, DPAT treatment, testosterone level, and sexual experience were incorporated into the model by allowing these factors to influence the hazard function. Finally, the parameters of the model were estimated using the maximum likelihood method of estimation (Greene, 2000).

3. Results

Table 1 provides general information on the number of subjects both overall and in each experimental condition that demonstrated (noncensored) or failed to demonstrate (censored cases) the response of interest. In general, the higher the number of censored to noncensored cases, the less likely survival regression analysis was able to yield reliable results.

Table 2 displays the results of reanalysis of latency to intromission/mounts and ejaculation using survival regression analysis; these results are presented along with the results of the original analyses on these data (Rowland and Houtsmuller, 1998). Because the reanalysis did not require construction of new variables, comparisons across studies involve similar though not necessarily identical variables.

3.1. Latency to first mount or intromission

Because mounts and intromissions stem from the same underlying motivational system and represent variations of a similar response system (attempt to mount and penetrate), no distinction was made between these responses for purposes of analysis. With respect to the latency to the first mount or intromission, the coefficient P was less than 1.0 (0.64), indicating a hazard function with a downward slope. In other words, the longer a rat continued without displaying a mount or intromission, the lower the probability that it would ever mount or intromit. For this variable, testosterone and DPAT effects were found but no experience effect (Table 2). Coefficients (not included in Table 2) indicated that testosterone decreased and DPAT increased the latency to the first mount/intromission.

3.2. Latency to first ejaculation

Results for latency to ejaculation indicated a coefficient P greater than 1.0 (i.e., 1.43), indicating a hazard function that slopes upward. That is, the longer a male rat continued without ejaculating, the higher the probability that it would ejaculate. As in the original analysis, reanalysis indicated an effect of testosterone, with both the moderate and high testosterone males showing shorter latencies than low tes-

Table 1
Noncensored and censored cases for subgroups within the sample on the variables latency to first mount/intromission and latency to ejaculation

Variable	Subgroup	Total n	Noncensored n	Censored n
Ejaculation	Overall	106	45	61
	DPAT	54	27	27
	Non-DPAT	52	18	34
	Experienced	52	27	25
	Inexperienced	54	18	36
	Low testosterone	38	1	37
	Med testosterone	37	16	21
	High testosterone	31	28	3
Mounts/intromission	Overall	106	93	13
	DPAT	54	45	9
	Non-DPAT	52	48	4
	Experienced	52	49	3
	Inexperienced	54	44	10
	Low testosterone	38	29	9
	Med testosterone	37	33	4
	High testosterone	31	31	0

Table 2
Comparison of effects (P values) of survival analysis and traditional ANOVA strategies in the analysis of male sexual response

	Ejaculation latency		Mount/intromission latency	
	Survival	ANOVA	Survival	ANOVA
Independent variable				
DPAT	.003	.002	.005	.006
Experience	.690	.817	.229	[a]
Testosterone[b]				
Med	.004		.001	[a]
High	.000	.001	.000	[a]
DPAT × Experience	.184	.066	[a]	[a]

Survival = reanalysis using survival regression; ANOVA = original analysis using ANOVA on constructed variables.

[a] Analyses not undertaken in the original or present study.

[b] For survival analysis, the Med and High testosterone groups were significantly different from the Low testosterone group; post-hoc analysis comparing Med and High testosterone groups was not carried out. For ANOVA, the Med and High testosterone groups were significantly different from each other; the Low testosterone group was not included in the analysis.

tosterone males. A DPAT effect was also identified such that DPAT-treated males had shorter latencies to ejaculation. No effect for experience was found.

A second survival regression model for latency to ejaculation included the interaction term "DPAT by experience," the rationale being that the effect of DPAT on ejaculatory latency would be more pronounced in experienced males. In contrast with the original analysis showing a near significant (.06) interaction effect, survival analysis yielded a probability of .18 for this interaction variable.

3.3. Determination of optimal test length

Table 3 provides estimations of descriptive parameters relevant to testing session length. In using survival regression analysis, an overall testing session length necessary to capture 50% or more of the ejaculations could be established, as well as optimal lengths for males under the various experimental conditions (i.e., low, medium, or high testosterone; DPAT or saline; experienced or naive). Additional analyses generated optimal testing lengths for those males exhibiting a sexual response of any sort (i.e., for males that mounted or intromitted at least once) during the behavioral tests.

4. Discussion

Survival regression analysis provides a suitable way of handling data from experiments having censored cases, as typically occurs in many experimental behavioral studies. The example provided here illustrates a specific application of survival regression analysis to the study of male sexual behavior in the rat. Although the conclusions based on the reanalyzed data differed only moderately from those suggested in the original analysis (Rowland and Houtsmuller, 1998), they can be drawn with the confidence that bias from dropping cases and distortion from constructing new variables did not occur. Furthermore, this reanalysis did reveal that when the low testosterone group was retained in the analysis, the marginal DPAT × Experience interaction seen in the original analysis was not apparent. Although this interaction was suggested in the response patterns of males with moderate testosterone, the disparity between the original and present analyses highlights one of the problems inherent in constructing latency variables based on overall test session length — a strategy used not only in our original analysis but in other studies as well (e.g., De Jonge et al., 1989). Specifically, for the original analysis, a substantial number of nonejaculating males were assigned a latency of

Table 3
Testing session length (in minutes) required to capture 50% or 75% of ejaculations and mounts/intromissions

Variable	Group	50%	75%
Ejaculation	All males	87.1	141.2
	Intromitting males	67.5	108.8
	DPAT males	61.6	102.1
	Non-DPAT males	[a]	[a]
	Experienced males	55.1	83.9
	Inexperienced males	[a]	[a]
	Low-testosterone males	[a]	[a]
	Med-testosterone males	57.6	96.0
	High-testosterone males	19.8	30.7
Mounts/intromission	All males	5.5	15.9
	Intromitting males	3.5	8.6
	DPAT males	9.1	25.9
	Non-DPAT males	3.2	9.5
	Experienced males	3.8	11.3
	Inexperienced males	7.8	20.4
	Low-testosterone males	17.9	40.2
	Med-testosterone males	3.1	12.1
	High-testosterone males	2.4	5.4

[a] Unreliable estimate due to the high proportion of censored cases.

45 min (based on the overall test session length), a procedure that artificially decreased within-group variance and, in so doing, may have augmented the probability of a Type I error. In the reanalysis using survival regression, this potential bias was circumvented.

Of significance to studies investigating the sexual behavior of male rats, survival regression analysis yields a hazard function that indicates the conditional probability of a behavior, given that it has not occurred by the end of the testing session. Specifically, in the overall sample, the longer an intromissive response failed to occur, the lower its probability of occurrence became, whereas the longer ejaculation failed to occur, the greater its probability of occurrence. Although this latter conclusion may seem counterintuitive for nonintromitting males (i.e., the probability of ejaculation in nonintromitting males should *not* increase with the passage of time), the overall hazard function reflects the larger pattern of the entire sample, in which 93 of 106 males (88%) intromitted. Although not examined as part of this analysis, post-hoc analyses could have been used to differentiate the conditional probabilities for these responses within various subsets of experimental groups (e.g., intromitting vs. nonintromitting males).

Survival regression analysis also enabled establishment of optimal test session lengths. For example, to capture 50% of the ejaculations for all males would have required a test session of 87 min. To capture 50% of ejaculations of males exhibiting any sexual behavior would have required a test session of 67 min, and to capture 50% of ejaculations of sexually experienced males would have required a session of 55 min. Although these estimates are specific to the testing conditions and strain of rats (Wistar) used in this study, they nevertheless suggest that studies using 15–30-min test sessions may have sampled only a subpopulation of highly sexually active males, thereby reducing generalization of findings to the overall population.

As with any analytical procedure, survival regression analysis has limitations. Specifically, for subgroups showing a high proportion of censored relative to noncensored cases (as, for example, occurred in the low testosterone group where few males ejaculated; Table 1), information sufficient for reliable estimates was lacking. However, as long as the number of censored cases did not exceed about 60% of the cases, we were able to generate fairly reasonable estimates of the time parameters for an optimal test session length. In our view, it would be difficult to find any estimation procedure (or statistical test) that could generate reliable estimates and make valid inferences for subgroups where the number of censored cases (nonresponders) exceeds that level.

More generally, compared with conventional (nonparametric) survival analysis, survival regression analysis requires that the researcher impose a structure on the data by assuming a probability distribution for the dependent variable. In so doing, the researcher risks imposing an inappropriate structure on the data and/or drawing inappropriate inferences from the censored portion of the distribution. However, the very power to draw such inferences is enabled only by the fact that survival regression analysis goes beyond the empirical approach of the Kaplan Meier estimator, this latter method relying on the data alone to obtain estimates of survival and hazard rates. Furthermore, in survival regression analysis, description of the random variable using the Weibull distribution, which allows the hazard rate to increase, decrease, or remain constant as a function of the passage of time, affords a flexible approach that under most circumstances yields reliable estimates and easy-to-interpret results. Of course, as with any inferential statistical procedure that offers advantages by assuming a continuous dependent variable, the appropriate use of survival regression analysis requires a priori understanding of the behavior of the variables and their measures within the data set.

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