Clutch Effects Explain Heart Rate Variation in Embryonic Frogs (Cave Coqui, *Eleutherodactylus cooki*)

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ABSTRACT

Few physiological studies to date have focused on whether variation among sibling groups during development can account for often large, intraspecific physiological variation. In this study, we measured heart rate in the direct-developing frog *Eleutherodactylus cooki* throughout its embryonic development and examined heart rate variation among egg clutches comprising from 10 to 40 eggs. Clutches were collected in the wild in Yubucoa, Puerto Rico, and individual eggs were maintained under equivalent conditions in the lab. Heart rate showed large increases during development, rising from about 40 beats min^{-1} in the earliest stages to about 110 beats min^{-1} at hatching. The effect of stage (averaged across clutches) was highly significant ($P < 0.001$). However, repeated-measures MANOVA also revealed that there were highly significant effects on heart rate associated with both clutch (variation among clutches averaged across development; $P < 0.001$) and clutch-stage interactions (differences among clutches in the developmental change in heart rate; $P < 0.0001$). These effects and interactions reveal that throughout development, heart rate in siblings is much more similar than in nonsiblings and that sib groups follow different heart rate trajectories during their development. Collectively, these data indicate that "clutch effects" caused by genetic and/or maternal influences can strongly affect patterns of heart function during development within cave coqui populations. This phenomenon also occurs in bird eggs and armadillo neonates, suggesting that physiological variation attributable to clutch effects might be a widespread phenomenon in vertebrates.

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Introduction

Investigations of physiological development traditionally have not controlled for or examined so-called clutch effects, or sibling effects. Yet they can be an interesting, large, and potentially confounding source of variation (Packard and Packard 1993). Such variation can easily obscure experimental treatment effects or inherent differences between different populations. Yet our scrutiny of physiological development articles reveals that the large majority of experimental designs do not control for clutch effects, with either all embryos or larvae coming from a single set of parents (i.e., a single clutch) or, alternatively, coming from multiple parents with no reported control for clutch as a treatment effect. Developmental studies are beginning to increase their focus on sources of physiological variation among offspring of different or similar parents (e.g., Burggren et al. 1994; Bouchard et al. 1998; Schwabl 1999; Steyermark and Spotilla 2000). With respect to variation in cardiovascular function, studies of heart rate in developing bird embryos (Burggren et al. 1994; Tazawa et al. 1994) and clonal groups of neonatal armadillos (order *Xenarthra*; Bagatto et al. 2000) have shown that intraspecific differences in physiological performance during development can be profound. Parental effects (maternal and paternal), environmental effects, and genetic factors, possibly in combination, may account for such a phenomenon (Bernardo 1996; Burggren 1999).

That a clutch effect in heart rate occurs during development in both birds and mammals suggests that it may be a general characteristic of vertebrates. In addition, variation in developmental trajectories of heart rate may vary among clutches and targeted or compensatory development (Atchley 1984) such that trajectories converge on a less variable hatching or juvenile heart rate. Both developmental patterns indicate variation in the timing and magnitude of underlying physiological processes that must be characterized to understand cardiac development. In this study, we test for differences among clutches, developmental stages, and clutch-stage interactions in the cave coqui, *Eleutherodactylus cooki*, a direct-developing amphibian of Puerto Rico. The genus *Eleutherodactylus* is the single largest

vertebrate genus in terms of species number and is of interest because its species are direct developing; that is, they undergo development to the adult morph within their large, terrestrially laid eggs. Females do not participate in parental care, so there are no postdeposition maternal effects on ova development. *Eleutherodactylus cooki* was of special interest because this frog occupies subterranean caverns in which variation in thermal environment is small (<1°C) and humidity levels are consistently high (above 85%). Consequently, environmental differences are minimal among egg deposition sites (Rogowitz et al. 2001). Because the embryos are large and translucent throughout most of their development within the egg, this genus readily lends itself to noninvasive visual observation of heart rate over an extended period of development. Moreover, Eleutherodactylids typically lay distinct, isolated egg clutches, facilitating an analysis of intra- and interclutch physiological variation. Collectively, these traits make this genus useful animal models for developmental physiology (Burggren 2000).

The purposes of this study are (1) to extend a literature showing strong cardiovascular, physiological clutch effects to yet another vertebrate family (Amphibia), thereby further supporting its probable vertebrate-wide distribution and (2) to provide this article as a case study on how ignoring clutch effects can obscure other interesting sources of experimentally induced or ecologically based variation.

Material and Methods

Collection of Eggs

Eleutherodactylus cooki egg clutches were collected from three interconnected caves located in the forest of Yubucoa, Puerto Rico, during April 1996. Physical attributes and ambient conditions of these caves have been described previously (Rogowitz and Sánchez-Rivoleda 1999; Rogowitz et al. 1999, 2001). No significant environmental differences existed between these caves, and cave source was not considered as a source of variation. Fourteen egg clutches consisting of 320 embryos at stages 5, 6, and 10–15 (near hatching) were collected in the field and transported to the University of Puerto Rico in separate numbered, vented plastic ziploc bags containing moist paper towels. All eggs were separated, weighed, and then staged according to Townsend and Stewart's (1985) work on embryos of *E. coqui*.

All eggs from all clutches were maintained and treated identically insofar as possible and, in most cases, incubated concurrently in the same carefully controlled laboratory environment. In every case, a maximum of 4 h elapsed between collection and placement of the eggs in hydrated incubation conditions. Individual eggs were placed in numbered 1.5-mL centrifuge tubes with a moist paper towel and a perforated lid for air ventilation. Each tube containing an egg from the same clutch was then placed in a clutch-specific, numbered plastic ziploc bag that was ventilated with humidified air and floated in a temperature-controlled water bath at 23° ± 0.1°C, which was very similar to the cave temperature at the time of collection and to the mean annual cave temperature (Rogowitz and Sánchez-Rivoleda 1999). Because of the near darkness found in the natural habitat from which eggs were collected, an opaque cover was placed over the water bath to mimic natural conditions. All physiological measurements were carried out at 23.0° ± 0.4°C. In general, because the thrust of this study was to look for clutch-specific differences, great pains were made to rear all embryos in all clutches under as similar conditions as possible. However, our methodology does not eliminate the potential for very small variations in humidity and temperature between individual centrifuge tubes, so the experimental design and statistical analysis necessarily confounds clutch effects and any "bag effects" that might exist.

Finally, because the local populations of this species are threatened, all newly hatched *E. cooki* were carefully returned to the locations in the caves in which they were collected as eggs.

Measurement of Heart Rate

The egg case and embryonic body wall of *E. cooki* are transparent throughout all stages of cardiac development. Thus, at all stages of development, heart rate (f_H) could be determined using a dissection microscope (magnification ×10 to ×20) with substage fiber-optic illumination. Eggs were transferred to a watch glass that was then placed on the microscope stage. To record any possible changes during the measurement period, temperature was monitored at the surface of the egg using the 0.4-mm tip of a thermocouple probe attached to a PhysiTemp BAT-12 temperature monitor. (Perforation of the egg itself for embryonic body temperature measurement is not possible because of the immediate and complete collapse of the eggs when punctured.) Temperature did not vary by more than 0.4°C over the period of all heart rate measurements, and typically temperature varied by no more than 0.2°C during measurements in single embryos. Two 30-s sample periods separated by 1 min were averaged to determine f_H of an embryo at a given stage.

Data Analysis

Heart rate data are presented as mean values ± 1 SE. Changes between developmental stage, variation among clutches, and differences in the developmental change in heart rate among clutches were tested with stage effects, clutch effects, and stage-by-clutch interactions in a repeated-measures MANOVA (SAS 6.07; SAS 0000). Because not all individuals were measured at every developmental stage, we analyzed two distinct sets of data to include as much variation across development and as much variation among clutches as possible. The first analysis includes three clutches with heart rate data for all individuals (N = 100) for developmental stages 6–11, 13, and 14. The second analysis includes five clutches with heart rate data for all in-

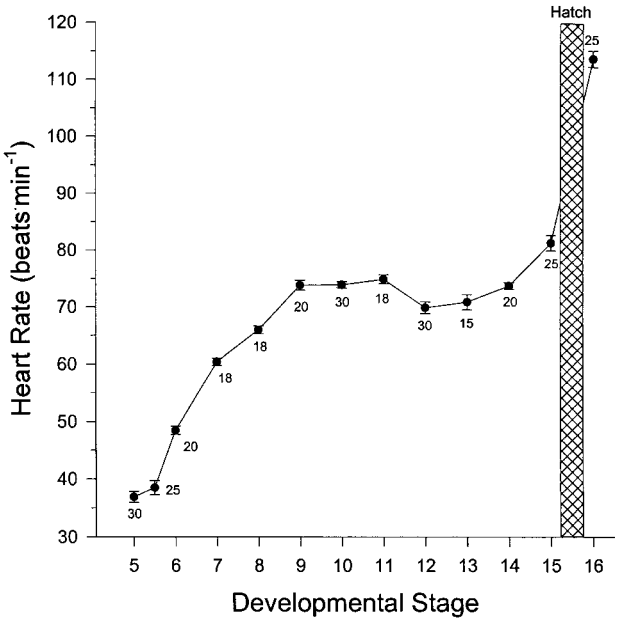

Figure 1. Heart rate at 23° ± 0.1°C as a function of development in embryonic *Eleutherodactylus cooki*. Crosshatched area indicates period of hatching. Mean values ± 1 SE. Numbers of individual observations contributing to each mean are indicated. Although development heart rate (f_H) data were collected from various stages in a total of 14 clutches, we have included f_H data from only those seven clutches comprising complete data sets (defined as data from six or more developmental stages).

Results

Figure 1 indicates mean f_H for seven clutches ($N = 294$) for which we acquired a relatively complete data set (defined as measurements on six different stages). The mean f_H of embryonic *Eleutherodactylus cooki* at stage 5 (days 4 and 5) was approximately 36–38 beats min^{-1} at 23°C (Fig. 1). Mean f_H then climbed to 70–76 beats min^{-1} during the next five stages, where it leveled off between stages 10 and 11. Heart rate then decreased slightly in stage 13 and increased again in stage 14. Mean f_H shows a final sharp increase in the few stages before hatching, reaching a peak of about 115 beats min^{-1} in hatchlings.

Repeated-measures MANOVA indicated that the overall effect of stage on f_H was highly significant (Table 1; $P = 0.0001$). The profile analysis of the difference in f_H between adjacent stages revealed significant increases in f_H from stage 6 to stage 10, no change between stage 10 and stage 11, a significant decrease from stage 11 to stage 13, and a significant increase in f_H from stage 13 to stage 14 (Table 2).

Highly significant variation in patterns of change in mean f_H during development existed among clutches (Table 1; Fig. 2) even though all eggs were separated and all clutches were maintained and measured in an identical fashion. The repeated-measures MANOVA of f_H changes tested for the significance of (1) stage effect (stage-to-stage differences in f_H averaged across clutches), (2) clutch effect (clutch-to-clutch differences in f_H averaged across developmental stages), and (3) clutch-stage interaction (differences in developmental change in heart rate

dividuals ($N = 49$) for developmental stages 9–11 and 13. We report P values from exact F-tests and Wilks's Lambda approximate F-tests in the overall MANOVA. However, all alternative test statistics yielded the same conclusions.

To determine the critical stages of development where there are significant changes in heart rate and significant variation among clutches, we used a profile transformation in the repeated-measures MANOVA. We constructed contrast variables of the difference in heart rate between each pair of adjacent stages (seven contrasts for the analysis of three clutches and three contrasts for the analysis of five clutches). Significant change in heart rate between adjacent stages was tested with the mean of the difference between stages. Significant variation among clutches in the magnitude of change between adjacent stages of development was tested with the effect of clutch on the difference between stages. We note that, traditionally, repeated-measures statistics are carried out with fixed time intervals between measurements. Yet in a developmental context, the choice of fixed time intervals over developmental stage as the independent variable is somewhat arbitrary because development is as accurately (or more accurately) described by morphological and physiological advancements than by time alone.

Table 1: Summary of repeated-measures MANOVA of heart rate through development for two sets of data

Effects Tested/Clutch Comparisons Performed (Clutch Number)	df	P Value
Stage effect:		
Three-clutch analysis (clutches 1–3)	7	.0001
Five-clutch analysis (clutches 1–5)	3	.0001
Clutch effect:		
Three-clutch analysis (clutches 1–3)	2	.007
Five-clutch analysis (clutches 1–5)	5	.0001
Clutch-stage interaction:		
Three-clutch analysis (clutches 1–3)	14	.0001
Five-clutch analysis (clutches 1–5)	15	.0001

Note. See "Material and Methods." The analysis of three clutches includes heart rate data for all individuals ($N = 100$) for developmental stages 6–11, 13, and 14. The analysis of five clutches includes heart rate data for all individuals ($N = 49$) for developmental stages 9–11 and 13. The between-subjects effect (clutch) tests variation among clutches averaged across stage. The within-subjects effect (stage) tests variation among stages averaged across clutch. The interaction of clutch-stage tests the degree to which the change in heart rate between developmental stages differs among clutches. The P value is from a Wilks's λ approximate F-test in the overall MANOVA.

Table 2: Contrasts of heart rate between adjacent stages of development from a profile transformation in the repeated-measures MANOVA

		P Value						
Clutch Comparisons Performed	df	Stages 6–7	Stages 7–8	Stages 8–9	Stages 9–10	Stages 10–11	Stages 11–13	Stages 13–14
Mean:								
Three-clutch analysis (clutches 1–3)	1	.0001	.0002	.039	.014	.349	.0005	.048
Five-clutch analysis (clutches 1–5)	1	…	…	…	.0008	.178	.0001	…
Clutch:								
Three-clutch analysis (clutches 1–3)	2	.002	.0001	.153	.0001	.192	.0005	.0001
Five-clutch analysis (clutches 1–5)	5	…	…	…	.0001	.0001	.0001	…

Note. The contrast variable is the difference in heart rate between each pair of adjacent stages (seven contrasts for the analysis of three clutches and three contrasts for the analysis of five clutches). The *F*-test of the mean tests for a change in heart rate between adjacent stages. The *F*-test of clutch tests for variation among clutches in the magnitude of the change in heart rate between adjacent stages. The *P* value is from an exact *F*-test for each contrast variable.

between clutches). As noted, highly significant effects of stage occurred throughout ontogeny. Analyses of the effects of clutch on heart rate across all stages of ontogeny were also highly significant (Table 1) in tests of three or five clutches. The analysis of the three clutches for which the broadest data set existed showed that this effect was explained primarily by interclutch variation between stage 7 and stage 14 (as indicated in Fig. 2). Even more striking, there were significant clutch-stage interactions throughout ontogeny (Table 1). This variation among clutches in the magnitude of change in f_H indicates that egg clutches differ in the developmental timing of heart rate increases. The profile analysis contrasting heart rate in adjacent stages of development indicates that there were significant effects of clutch on f_H change between all stages of development except for stages 8 and 9 and stages 10 and 11 in the analysis of three clutches (Table 2, clutch).

To summarize these analyses, patterns of f_H change during development can be much more similar within clutches than among clutches, even as f_H changes greatly during development in all individuals. In addition, relatively complex among-clutch variation exists in the timing of f_H development. For example, clutch 3 at stage 9 had an f_H (about 88 beats min^{-1} with little variation within clutch) that was about 30% higher than clutch 6 at stage 9. However, by stage 10, f_H in clutch 3 had decreased slightly, while f_H in clutch 6 jumped by 25% from 60 to 75 beats min^{-1}, so that the variation in average levels was greatly reduced (Fig. 2).

Discussion

Analysis of the heart rate data for *Eleutherodactylus cooki* reveals that not only does heart rate vary as a function of development (stage) but that considerable variation also occurs among egg clutches of comparable developmental stage. That is, variation in heart rate among clutches is significantly greater than variation of siblings within clutches (Table 1). Moreover, the variation has a complex pattern. Rather than one clutch persistently having a higher or lower mean heart rate throughout development, different populations "trade places" with respect to the heart rate hierarchy (Fig. 2*B*).

These data provide a clear example of rising above "the tyranny of the golden mean" (Bennett 1987, p. 148) encapsulated in Figure 1. By examining individual clutches, we may be able to see variations between clutches that could have ecological and physiological relevance but that would not be evident unless data were collected and identified by clutch rather than creating a single mean value for heart rate during development. In this context, it is interesting to speculate about whether a clutch effect would have been evident in a species of the same genus (*E. cooki*; Burggren et al. 1990). If only we had thought a decade ago to record our data in a way that would have allowed us to identify developmental patterns other than those of the species.

The greater similarity of heart rate in siblings compared with nonsiblings has now been demonstrated in the eggs of birds as a clutch effect (Burggren et al. 1994) and in clonal neonates of armadillo litters as a sibling effect (Bagatto et al. 2000). With this additional demonstration of a clutch effect in the eggs of an anuran amphibian bringing to three the number of vertebrate classes sharing this property, we suggest that this might be a cardiac physiological phenomenon occurring broadly throughout the vertebrates and worthy of more study.

The most striking result of this analysis is the highly significant differences among clutches in developmental trajectories. The timing and magnitude of heart rate increases and decreases varied among clutches, yet there is not a clear pattern of early heart rate increases, balanced by smaller changes in later stages or vice versa, that would indicate compensatory development. However, in all analyses, heart rate significantly decreased between stages 11 and 13, and the largest decreases in mean heart rate were seen in clutches that had the highest heart rate at

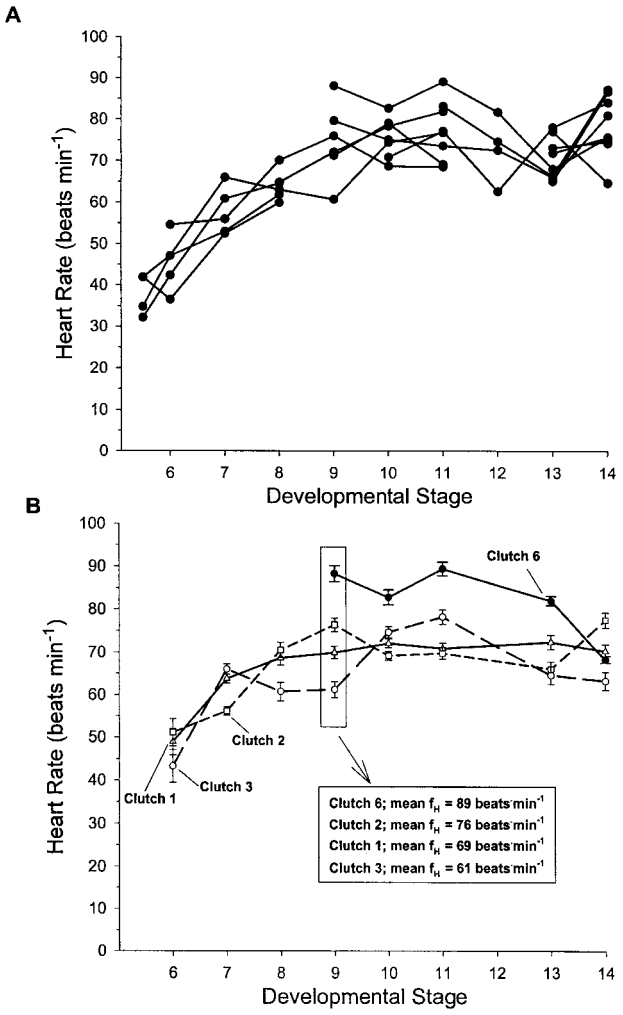

Figure 2. Between- and within-clutch heart rate variation in embryonic *Eleutherodactylus cooki*. Each plotted point is mean development heart rate (f_H) at 23° ± 0.1°C of a single clutch at a single developmental stage. *A*, All mean f_H data collected from a total of 14 clutches, including those for which fewer than six different developmental stages were measured. Standard errors have not been plotted to allow easier identification of specific clutches over development. *B*, To emphasize the magnitude of potential variation, mean f_H ± 1 SE are plotted for four clutches showing the most extreme between-clutch differences in mean f_H. Note that at stage 9, for example, there is extremely little within-clutch variation, yet there is large between-clutch variation, with clutch 6 exhibiting an f_H nearly 50% higher than clutch 3. Note also how clutches exchange relative positions in the hierarchy of f_H at different developmental stages, the source of the clutch-stage interaction in Table 1.

stage 11 (Figs. 1, 2). This result suggests that there may be compensation in physiological control or adjustment of heart rate toward a stage-specific target. Measurements of more clutches are required to characterize fully the nonlinear developmental changes in heart rate and differences in timing. Yet the fact that significant variability in trajectories could be detected with just six clutches indicates that potentially multiple sources of variation remain to be uncovered that could provide insight into the development of cardiac physiology and the importance, if any, of these different developmental trajectories.

While our clear identification of a clutch or sibling effect on heart rate is one thing, unequivocally identifying the mechanism that produces it is quite another. Common environment, maternal (or paternal) effects, and genetic effects (Arnold 1987) are three possible causes for similar phenotypes among full siblings (progeny of a single male and female). Common environment effects include the influences on phenotype expression due to environmental circumstances shared by siblings, including egg environments and experimental conditions. The effects of different cave locations before egg collection (eggs were collected from three distinct locations in proximity to each other) might have contributed to common environmental variation subsequently measured among clutches in the lab. Yet microclimate with respect to temperature, humidity, oxygen, light intensity, and so forth is highly uniform in caves occupied by *E. cooki*. Specifically, there is no significant thermal or hydric variation evident over a horizontal or vertical gradient (Rogowitz and Sánchez Rivoleda 1999). Thus, we expect that such influences would be small and probably diminish through development. We also went to great lengths to standardize temperature, humidity, and lighting during both incubation and measurement. Because eggs were isolated and clutches were reared in the same water bath at the same time, any phenotypic variation due to experimental differences should be distributed randomly across all clutches and thereby would not contribute to common environmental mechanisms.

Maternal and paternal effects on offspring phenotypes are a widespread and important source of phenotypic variation for many traits (Arnold 1987; Mousseau and Dingle 1991). Such effects on eggs and embryos may be due to differences in expressing of the paternal genotype or differences in paternal condition (see Bernardo 1996 for general discussion and Burggren 1999 and Burggren et al. 1994 for the relevance of maternal effects in physiology). In most *Eleutherodactylus* frogs, including *E. cooki*, male parental care occurs, and females have no influence on eggs after oviposition. Males press their abdomen over clutches (to hydrate eggs, apparently), yet this does not affect egg viability in moist environments such as those occupied by *E. cooki* (Burrowes 2000). Males also participate in combat to protect clutches from predators, which affects egg survival but should not affect development. Maternal effects in this species probably derive primarily from variation in the extent of yolk provisioning. Maternal effects have been mimicked by removing or adding yolk to the eggs of lizards, resulting in small or larger hatchlings (Sinervo et al. 1992). Although the duration of physiological development of ova probably depends on specific causal links between paternal and offspring phenotypes, we expect paternal effects to diminish through development.

Genetic variation is the third potential cause of phenotypic

variation among clutches. Given that genetic variation or heritability has been detected for a wide variety of physiological, behavioral, and morphological traits (Bennett 1987; Mousseau and Roff 1987; Roff and Mousseau 1987), a genetic explanation of the clutch effect in *E. cooki* is perhaps most parsimonious. Unlike common environment and paternal effects, genetic influences can be expected to contribute to physiological variation among clutches throughout development, including differences in timing of gene expression that could contribute to the clutch-by-stage interaction or variation in heart rate that was a prominent feature of our analysis. As just one example of a potential developmental mechanism, genetically regulated expression of proteins that form the ion channels in the cardiac pacemaker cells could vary in quantity, in quality, and in timing of appearance in different genotypes in a population.

It is interesting to speculate that the observed heart rate differences among clutches may have lasting consequences because Rogowitz and Sánchez-Rivoleda (1999) showed a significant individual variation in metabolic rates of adult *E. cooki* during rest and activity. Ultimately, the physiological variation among clutches in early development may result from the influences of sexual selection (differential success of egg clutches guarded by different males; Burrowes 2000), natural selection in cave environments, or the effects of genetic drift in isolated cave populations.

Data from this study add to emerging information on important clutch effects in water relations in developing reptiles (Packard and Packard 1993), heart rate in bird embryos (Burggren et al. 1994), and a variety of both morphological and physiological features in neonatal armadillos (Bagatto et al. 2000). Future studies must demonstrate whether environmental, maternal, or genetic effects (or a combination of all three) are producing this pronounced clutch effect on heart rate in the embryos of *E. cooki* or, indeed, the other above-mentioned species. Regardless of the underlying causes, the fact that there can be both large heart rate differences at any given stage in *E. cooki* as well as that clutches may exchange places in the "heart rate hierarchy" between stages (Fig. 2*B*) illustrates the unpredictable nature of the phenomenon. Consequently, we conclude that failing to control for clutch or sibling effects can lead to unintended variation in physiological data that could actually obscure real intent of a particular experimental protocol or procedure.

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