



Commentary

Pseudoreplication in use of predator stimuli in experiments on antipredator responses

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Stimuli of predators are commonly used in experiments by scientists in the field of animal behaviour to elicit responses from individuals of prey species (Caro, 2005; Ruxton, Sherratt, & Speed, 2004). This large body of experimental work has been instrumental in increasing our understanding of how prey behaviour is sensitive to predator stimuli, and also how prey might communicate to one another, or to a predator, as a result of detecting these stimuli. A key concern with many of these antipredator behavioural studies, however, may be the limited number of stimuli typically used by researchers in these experiments. The result might be considerable pseudoreplication in experimental design: repeated use of the same predator stimuli may be commonly and incorrectly treated as independent statistical samples (Hurlbert, 1984; Kroodsma, 1989, 1990; Kroodsma, Byers, Goodale, Johnson, & Chun, 2001). As a parallel to our current review, Kroodsma et al. (2001) reviewed studies of behavioural responses to conspecific vocal signal playbacks and found that well over half of the sampled articles contained studies where signals were played back multiple times but were statistically treated as if they were independent replicates. Here we review a sample of published experimental studies of

antipredator behaviour to assess the extent of pseudoreplication in these studies.

According to Hurlbert (1984, page 187), pseudoreplication is 'the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent'. In studies that treat nonindependent samples as being independent of one another, significance levels will typically be inaccurate due to the use of incorrect test statistics (Kroodsma, 1989, 1990; Machlis, Dodd, & Fentress, 1985; McGregor et al., 1992). Increasing the independence of stimuli used in experiments that involve presenting those stimuli to subjects would increase an investigator's ability to generalize results. Many authors have suggested designs that prevent pseudoreplication in a range of taxa and experimental approaches (Hurlbert, 1984; Kroodsma, 1990; Millar & Anderson, 2004; Waller, Warmelink, Liebal, Micheletta, & Slocombe, 2013). However, these arguments to avoid pseudoreplication have not been applied to every experimental approach in animal behaviour, let alone every modality in animal communication and perception.

Decades ago, the most common form of experimental replication was 'simple pseudoreplication'. If only one exemplar has been used for all of the trials within a study, the study suffers from simple pseudoreplication. For example, consider a test of a

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hypothesis that prey individuals increase their vigilance when they detect the call of a predator. Imagine the researcher plays a single call of the predator to 20 different individuals and compares those responses to a single control sound (such as white noise) played back to another 20 different individuals. If the researcher then statistically tests 20 predator responses against 20 control responses, he or she will have committed simple pseudoreplication. This person intends his or her inference space to be 'predator calls' compared to 'controls', but can safely only interpret the results to be limited to the two specific stimuli played back in the experiment. This issue has become less common in recent years in playback studies (Kroodsma et al., 2001), but has been replaced by the subtler problem of sacrificial pseudoreplication.

Sacrificial pseudoreplication is described by Hurlbert (1984, page 205) as occurring 'when an experimental design involves true replication of treatments but where the data for replicates are pooled prior to statistical analysis... or where the two or more samples or measurements taken from each experimental unit are treated as independent replicates'. In most experimental studies of antipredator behaviour, what this typically means is that more than one exemplar is present, but not enough to truly replicate the study. As one example, imagine a researcher uses five different taxidermy mounts of a hawk six different times each, for 30 total presentations to different focal subjects, and assesses subjects' responses to the hawk stimulus compared to their responses to a control stimulus. If the researcher then statistically tests the effect of the 'hawk' stimulus against the control stimulus with 30 subjects as the unit of analysis (rather than the 5 taxidermy mounts as the unit of analysis), he or she will have committed sacrificial pseudoreplication. Although sacrificial pseudoreplication is methodologically stronger than simple pseudoreplication (it does, after all, have greater independence in the stimuli used), it can still lead to inaccurate results, as the true value of the effect with regard to the intended inference space cannot be accurately estimated (Kroodsma et al., 2001).

The last form of replication is true replication. While not always easy to achieve, truly replicated designs are necessary to permit correct interpretations in the inference space we typically wish to understand (Kroodsma et al., 2001). The most straightforward instance of true replication would involve a researcher observing statistically independent samples (each data point represents a different individual or group being tested) and using independent stimuli for each trial (each data point comes from a trial using a unique stimulus that is not used again). For example, if a researcher tests whether chemical cues of a particular felid predator affect vigilance rates of individuals of a prey species, true replication would involve each individual of the prey species being tested with chemical cues obtained from unique individuals of the felid species. In other words, every trial not only has a new subject, but also a new stimulus. By employing true replication, researchers can avoid pseudoreplication, report an accurate threshold of significance and better avoid type I errors (Hurlbert, 1984).

For this review we examined the extent of pseudoreplication in animal behaviour studies in which researchers experimentally presented stimuli of predators to elicit behavioural responses from individuals of prey species. The rationale behind this approach is typically to use the stimulus as a 'stand-in' for the predator, or to test the stimulus per se to determine specifically how prey respond to that predator stimulus (e.g. a sound) compared to another predator stimulus (e.g. visual cues), or to do both. Just as no two birds sing exactly the same song (Kroodsma, 1989), no two predators have exactly the same signals or cues.

In October–November 2014, we sampled studies in the University of Tennessee's Web of Science database (Thomson Reuters), using the search terms '(anti-predat* and behav* and stimul*)', in

which the * is a wild card character that includes all words with the base search term, but that have unique endings, such as 'behavior', 'behaviour', 'behaviors', 'behaving', etc. for 'behav*'. The review returned 150 references from the primary science literature. We read the abstract of each of the 150 references to determine which described experimental studies of antipredator behaviour (we did not include reviews or naturalistic observational studies in our analysis). We reviewed the methods and results sections of each article deemed 'experimental' to determine whether or not stimuli were pseudoreplicated. Studies were also coded by modality (whether the stimuli tested were acoustic, chemical, visual or multimodal). We found only a few studies that tested tactile or vibrational stimuli of predators, and we did not include these in our analysis. We only assessed studies that experimentally manipulated (presented) predator stimuli, and so did not consider studies of conspecific alarm signals or cues. Ultimately, we evaluated 71 articles to determine the extent of pseudoreplication in stimulus use (see [Supplementary Material](#)). These articles were published in 41 different journals, indicating that our review covered a wide range of fields, approaches and research traditions. The most represented journals in the review had five articles each: *Behavioural Processes*, *Ethology* and *PLoS One*. We independently assessed inter-rater agreement for 30 randomly selected articles in our review, and we agreed on whether the study had pseudoreplication or true replication in 29 of those articles.

We found that 95% of the studies we examined (68 of 71) were pseudoreplicated. No one modality seemed less likely to commit pseudoreplication, using exact 95% confidence intervals for the binomial probability (Conover, 1999; Fig. 1). In our sample, no more than one study in any particular modality had true replication. We are reluctant to criticize individual pseudoreplicated studies of others, especially given that two of the published articles in our review that were pseudoreplicated were coauthored by one of us (T. M. Freeberg). So, we briefly focus on these two studies here. Bartmess-LeVasseur, Branch, Browning, Owens, and Freeberg (2010) presented painted plastic hawk models at two different distances from a feeding station being used by small songbird species. Data on seed taking and calling were obtained from 15

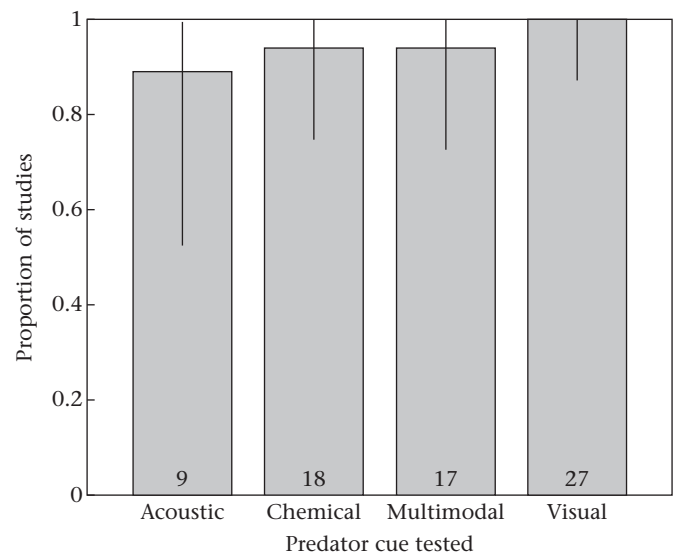


Figure 1. The proportion of pseudoreplicated studies in our review. The different modalities of predator cues are indicated on the X axis, with sample sizes of articles testing each predator cue type indicated in the graphic. Whiskers represent 95% confidence intervals for the binomial probability.

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