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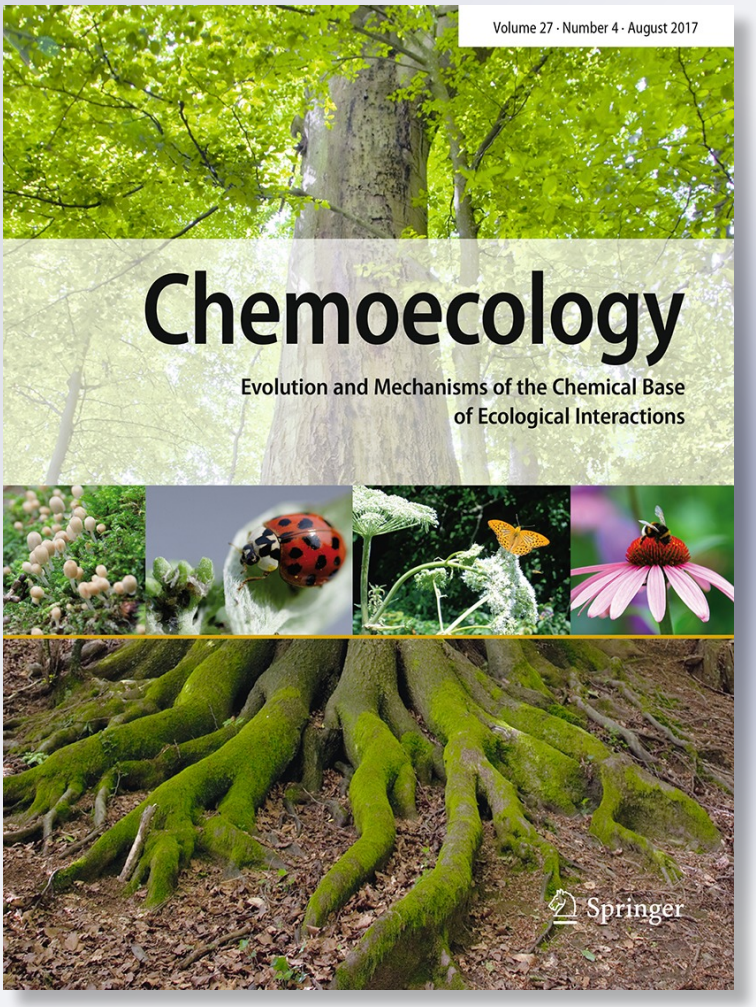

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COMMENTARY

Poison frogs, defensive alkaloids, and sleepless mice: critique of a toxicity bioassay

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Abstract In studies of defensive allomones, appropriate methods of presenting chemicals and measuring their deterrent effects on consumers are essential for understanding the contributions that chemicals make to the survivorship of potential prey. However, unnatural chemical presentations and/or ambiguous bioassay responses occasionally have left open questions on some allelochemical effects. This discussion critiques a toxicity bioassay of Neotropical poison frogs (Dendrobatidae), a group whose skins are known to possess a diverse array of bioactive alkaloids. The problematic bioassay entails injecting laboratory mice with the skin extracts of frogs and monitoring the time taken for mice to fall back to sleep to estimate extract toxicity, where longer latencies of sleep onset were claimed to reflect greater toxicity. Dendrobatids do not invasively deliver skin alkaloids to offenders, hence the method of injecting mice with skin extracts does not correspond to the frogs' natural defense mechanisms. Neither does the injection of frog extracts permit gastrointestinal deactivation or clearance mechanisms that may reduce the bioavailability of toxins. Whether or not increased sleep latencies induced by injected skin extracts reflect toxicity, irritability or other effects, the ultimate protective value for frogs of prolonging the wakefulness of mice (or relevant predators) is unclear. Defensible bioassays entail both modes of chemical delivery consistent with those by which would-be predators normally encounter chemicals and quantified measures of responses by predators that detract from their success.

Keywords Alkaloids · Chemical defense · Dendrobatidae · Poison frogs · Toxicity bioassay

Introduction

Chemicals that deter consumers play pivotal roles in interactions throughout nature. Appropriate methods of presenting chemicals and measuring their deterrent effects against predation are essential if the contributions that chemicals make to survivorship are to be properly evaluated and their ecological significance understood. Documentation throughout the literature of the repellence of predators or their post-attack rejections of prey and prey-derived chemicals attests to the use of defensive allomones in many interactions. However, unnatural chemical presentations and/or ambiguous bioassay responses occasionally have left open questions on some allelochemical effects. Here, I discuss these problems arising in investigations of Neotropical poison frogs (Dendrobatidae), a group widely cited in discussions of chemical defense and a quintessential exemplar among vertebrates for the dietary sequestration of defensive chemicals (Savitzky et al. 2012).

Dendrobatids and their alkaloid arsenal

The Dendrobatidae contains ca. 180 species of small frogs that occur in leaf litter to forest canopy habitats from Nicaragua south through northern South America to Bolivia (Frost 2017). Many dendrobatids are brightly colored, an aposematic feature related to the noxious skin chemicals

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that they primarily sequester from their diet of ants, mites, and other arthropods. More than 550 alkaloids representing over 20 compound classes have been isolated from the skin of dendrobatids (Saporito et al. 2012). Some of these compounds, tested singly, exhibit toxicity in investigations of their pharmacological mode of action, e.g., binding to types of neurotransmitter receptors (Santos et al. 2016). Several compounds, notably pumiliotoxins, are known to act as contact toxins against insects, penetrating their cuticle and inducing convulsions, leg autotomy, immobility, and death (Weldon et al. 2006, 2013).

The bioassay and its purported significance

In a series of studies of anti-predator defense and sexual signaling of dendrobatids, Cummings and colleagues compared the skin toxicities of different frog species or morphs (Darst and Cummings 2006; Darst et al. 2006; Maan and Cummings 2012; Cummings and Crothers 2013). To obtain extracts, frogs were euthanized by topically applying benzocaine to their head and venter, their skin was removed, and skin chemicals were extracted with methanol. The extracts were evaporated to dryness and the residues were re-dissolved in saline solution. The extracts from (usually five) individual frogs of each species or morph were then injected subcutaneously into (usually five) sleeping female mice (Harlan Laboratories, outbred strain CD-1), one frog extract per mouse, awakened for treatment. Saline solution and, in some experiments (Maan and Cummings 2012), the skin extracts of the Talamanca rocket frog (*Allobates talamancae*), an alkaloid-free dendrobatid, served as controls. The time taken for mice to fall back to sleep was used to estimate extract toxicity, where longer latencies of sleep onset were claimed to reflect greater toxicity. On the basis of this bioassay, referred to here as the sleepless mouse bioassay (SMB), the relative toxicities of frogs were assigned in purported demonstrations of (1) the correlation between frog toxicity and skin color brightness (Darst et al. 2006; Maan and Cummings 2012; cf. Daly and Myers 1967), (2) the establishment of learned avoidances by predators of Batesian mimic and model frogs (Darst and Cummings 2006; Darst et al. 2006), and (3) the interaction of natural and sexual selection in the evolution of aposematism and signaling related to female mate choice and male–male competition (Cummings and Crothers 2013).

Toxicity?

As Weldon and Burghardt (2015) and Bolton et al. (2017) pointed out, the bioassay developed by Cummings and colleagues is not clearly relevant to the natural predators of dendrobatids. One concern pertains to predator proxies being injected with skin extracts; although some amphibians, including anurans, possess skeletal elements that protrude through their integument and subcutaneously deliver skin toxins to offenders (Jared et al. 2015), dendrobatids do not. Hence, the method of injecting mice with skin extracts does not correspond to the frogs' natural defense mechanisms.

Neither does the injection of frog extracts permit gastrointestinal deactivation nor clearance mechanisms that may reduce the bioavailability of toxins (e.g., Gavhane and Yadav 2012). As Williams et al. (2002) stated, understanding the relationship between the effects of a toxin administered orally and through injection is critical if an oral dose is the exclusive natural mode of exposure. The skin and other organs of newts (*Taricha* spp.), for example, contain tetrodotoxin (TTX), an alkaloid that blocks voltage-gated sodium channels and inhibits the propagation of action potentials in muscles and nerves. North American deer mice (*Peromyscus maniculatus*) treated with newt skin extracts (Brodie 1968), and laboratory mice and garter snakes (*Thamnophis sirtalis*) treated with measured doses of TTX (Williams et al. 2002), required significantly greater oral doses of these substances to induce toxicosis than when they were administered by intraperitoneal injections. Williams et al. (2002) hypothesized that TTX administered orally may be degraded by stomach acidity.

Darst et al. (2006) cited Daly and Myers (1967) for establishing the skin extract injection of laboratory mice as "a standard protocol" for assessing frog toxicity. However, Daly and Myers (1967) injected mice to assess the lethality of different frogs' extracts, an outcome obviously more pertinent both to toxicity and to frog defense (mode of chemical delivery notwithstanding) than is latency of sleep onset. Maan and Cummings (2012) asserted that their method represents a "more sensitive toxicity assay" than that used by Daly and Myers (1967). Darst et al. (2006: 5856) had clarified that they actually "use the term 'toxicity' to refer to relative irritant effect of frog skin alkaloids".

Apart from toxicity and/or irritancy, the potential insomnolent effects of dendrobatid skin alkaloids cannot be dismissed as confounding the SMB. Many plant alkaloids, e.g., caffeine and nicotine, are documented stimulants, increasing the latency of sleep onset in mammals (Shneerson 2005). Nicotine also occurs in some dendrobatid skins (Weldon et al. 2013), as do many other (pharmacologically unscreened) alkaloids that may affect sleep activity. More fundamental to the mechanism(s) underlying sleep deferment, however, is the question of the ultimate protective value reflected by the SMB response, i.e., how does prolonging the wakefulness of predators enhance frog survivorship?

Unpalatability?

Unpalatability is the first line of anti-predator defense for many organisms. Referring to the SMB, Darst et al. (2006: 5852) stated, "Relative unpalatability of poison frog species was assessed by using a toxicity assay, because a quantitative assay for oral noxiousness does not exist". In fact, however, the unpalatability of dendrobatids to ants (Fritz et al. 1981; Stynoski et al. 2014a; Hantak et al. 2016; Murray et al. 2016), arachnids (Szelistowski 1985; Gray et al. 2010; Stynoski et al. 2014b; Hantak et al. 2016; Hovey et al. 2016; Murray et al. 2016), snakes (Lüling 1971; Brodie and Tumbarello 1978; Daly et al. 1978), birds (Darst and Cummings 2006; Darst et al. 2006), and mammals (Daly et al. 1978) is suggested by reports that these predators reject frogs after contacting them. Unpalatability may be quantified behaviorally as the number of predators or predator proxies that sample and reject different individual prey, prey tissues, or treated food versus controls; the latency to reject different prey items; or the amounts of chemically treated versus untreated food consumed. Bolton et al. (2017), for example, demonstrated population differences in the palatability of the frog (*Oophaga pumilio*) using a bioassay in which vinegar flies (*Drosophila melanogaster*) and ants (*Ectatomma ruidum*) were observed to drink less of sucrose solutions laden with frog skin extracts than of plain sucrose.

Maan and Cummings (2012: 3) stated that a basic assumption for the use of their SMB is that "subcutaneous injection induces responses that are representative of those generated when predators or parasites attack and/or ingest a frog". The responses by vertebrate predators associated with the ingestion of prey are regularly mediated, at least in part, by taste receptors in the buccal cavity, which might well be circumvented when stimulus chemicals are injected into subjects. Rodents and other mammals injected with some compounds may perceive blood-borne chemicals via intravascular taste (e.g., Bradley and Mistretta 1971); the involvement of intravascular taste in the perception of frog alkaloids, and its influence on sleep activity, however, remain unexplored.

Bolton et al. (2017: 286), commenting on the studies in which rodents have been injected with frog skin extracts, stated that "palatability and toxicity [i.e., lethality or induction of sleep latency] are not strongly related, and that toxicity measures may not be a reliable predictor of predator response to frog alkaloid defenses". The toxicity alone of dendrobatid skin alkaloids varies considerably, even among compounds that are dubbed "toxins". As Daly and Spande (1986) pointed out, the designation of some alkaloids as "toxins" is misleading; some compounds are not toxic in vivo or only weakly so in commonly used ex vivo assays. These alkaloids originally were isolated from frogs along with other alkaloids that are toxic and they were designated as toxins before their structures and individual activities had been elucidated. Hence, some frogs, e.g., *Andinobates* (*Dendrobates*) *bombetes*, are not particularly toxic despite possessing an abundance of alkaloids in their skin (Myers and Daly 1980). Nontoxic compounds may contribute to frog defense as distasteful agents, as postulated by Daly et al. (2005) for histrionicotoxins.

The unknown toxicities and palatabilities of hundreds of dendrobatid alkaloids, as well as population, temporal, and other sources of variation in skin alkaloid composition (Saporito et al. 2012), pose major challenges in constructing a predictive model of the noxious chemical properties of these frogs. Progress in this regard is not likely to derive from studies thus far using the SMB because compounds present in the injected skin extracts were not characterized qualitatively or quantitatively. Such chemical characterizations are important for replicability, especially given the aforementioned sources of variation observed in alkaloid skin content, as well as the small sample sizes of frog skin donors and predator proxies used in experiments. Aside from the general concerns recounted here, the failure by Daly (pers. comm.) to validate the results of the SMB in laboratory mice (NIH Swiss strain) with injected dendrobatid skin extracts further suggests that claims based upon it regarding the behavioral, ecological, and evolutionary significance of dendrobatid alkaloids should be re-examined.

Conclusions

Chemicals deployed against predators act through diverse mechanisms, overall, eliciting responses that, in various ways, preempt or interfere with attacks. As Nelson (1983: 34) wrote regarding sharks' responses to chemical deterrents, "Besides repellency, possible behavioral responses include rejection, regurgitation, feeding inhibition, irritation, aggression, and distress, incapacitation, or death. These responses are not mutually exclusive, and some substances would elicit more than one of them". Nelson's (1983) focus was on natural and synthetic anti-shark agents to be co-opted for human protection, but his statement underscores, in general, the prospects for diverse measures potentially adopted in bioassays of chemical deterrents. Along with diverse methods of deterrence come challenges in labeling responses according to the more general constructs they ostensibly reflect, e.g., toxicity, irritability, palatability or something else? In addition to quantified measures of responses by predators that detract from their success, defensible bioassays entail modes of chemical delivery consistent with those by which would-be predators normally encounter chemicals.

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