Active foraging for toxic prey during gestation in a snake with maternal provisioning of sequestered chemical defences

Yosuke Kojima and Akira Mori

Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8502, Japan

Many animals sequester dietary defensive compounds and incorporate them into the offspring, which protects the young against predation. One possible but poorly investigated question is whether females of such species actively prey upon toxic diets. The snake *Rhabdophis tigrinus* sequesters defensive steroids from toads consumed as prey; it also feeds on other amphibians. Females produce chemically armed offspring in direct proportion to their own level of toad-derived toxins by provisioning the toxins to their eggs. Our field observations of movements and stomach contents of radio-tracked *R. tigrinus* showed that gravid snakes preyed upon toads by actively foraging in the habitat of toads, even though toads were a scarce resource and toad-searching may incur potential costs. Our Y-maze experiments demonstrated that gravid females were more likely to trail the chemical cues of toads than were males or non-gravid females. These results showed behavioural switching in females and active foraging for scarce, toxic prey during gestation. Because exploitation of toads by gravid females results in their offspring being more richly endowed with prey-derived toxins, active foraging for toxic prey is expected to be an adaptive antipredator trait, which may enhance chemical defence in offspring.

1. Introduction

Parents reduce predation risk to their offspring with a variety of mechanisms, including provisioning offspring with chemical defences [1–6]. Parental endowment of chemical defences is a widespread strategy known from both invertebrates and vertebrates in marine and terrestrial ecosystems [1–6]. In certain groups of insects, the mechanism and ecology of chemical protection of offspring have received much attention (e.g. [3,7–9]), but there is comparatively little research on vertebrate systems, even though examples of vertebrate species that produce unpalatable or poisonous offspring are increasing [4,6,10].

Some defensive compounds in animals are synthesized by the defended taxa or their symbionts, but others are sequestered from environmental sources, such as diet [11,12]. In the latter cases, feeding ecology may be important because diet can determine the level of chemical defence [13]. The focus of this study is the feeding ecology of parents in the context of defensive provisioning. Natural selection may favour those females that actively accumulate and endow their offspring with more defensive chemicals when the concentration has an effect on offspring survival [3,7–9]. Although a study has reported mating-induced behavioural switching in females of a firefly, which is likely to facilitate acquisition of toxins used for chemical protection of offspring [14], few studies have addressed natural behaviour related to acquisition of defensive chemicals.

The Asian snake *Rhabdophis tigrinus* possesses unusual structures known as nuchal glands in the dorsal skin of the neck [15]. The fluid enclosed in the nuchal glands contains highly toxic cardiotonic steroids (bufadienolides) and is released when the snake is attacked [15,16]. Bufadienolides in the nuchal...
glands of *R. tigrinus* are ultimately sequestered from toads (*Bufo*) that are often consumed by the snakes, along with other amphibian prey [16]. Female *R. tigrinus* are capable of provisioning their offspring with sequestered bufadienolides via deposition in yolk and by transfer to oviducal eggs. The transfer of sequestered steroids can occur as late as 12 days prior to oviposition [6] and even following oviposition when those compounds are experimentally applied on the eggshell [17]. Females produce chemically armed offspring in direct proportion to their own level of toxins [6].

We test the hypothesis that gravid female *R. tigrinus* selectively forage for toads in a series of field observations and an experiment. Specifically, we investigate the following predictions: (i) gravid females more frequently use habitat where toads occur than do males; (ii) gravid females exhibit active foraging behaviour while in the habitat of toads; (iii) gravid females exploit toads more frequently than expected based on prey availability; and (iv) when choosing between a toad and a non-toxic frog, gravid females are more likely to choose a toad than are males or non-gravid females.

2. Material and methods

(a) Study site

The study site was approximately 2 km² in the Ashiu Forest Research Station (AFRS) of the Field Science Education and Research Center, Kyoto University, located in a mountainous area of northern Kyoto Prefecture, Japan (35°18’ N, 135°43’ E). Habitats in the study site include forests, grasslands, open riverbanks and a small paddy field (electronic supplementary material, figure S1; see [18] for more detail on the site).

(b) Study species

*Rhabdophis tigrinus* is an active forager and primarily feeds on anurans [19]. At AFRS, a dietary survey on snakes has been conducted over 30 years and has shown that *R. tigrinus* feeds mainly on non-bufoid frogs: *Rhacophorus arborus* and *Rhacophorus schlegelii*, which are locally abundant, non-toxic frogs, comprised 89% of the diet of *R. tigrinus*, whereas the toad *Bufo japonicus formosus* comprised only 0.9% of the diet [20]. Reproductive activity of *R. tigrinus* is seasonal: mating mainly takes place from late September to October, and females lay eggs around early July [21] (Y.K. 2014, personal observation). The snake is an income breeder [21], whereby females continue to consume prey during gestation.

(c) Habitat use of toads and relative abundance of anuran species

From April to November 2009–2013, we recorded the habitat of toads whenever we encountered them. We walked carefully, watching the ground during the daytime on non-rainy days. We walked throughout the study site, most frequently along the trail and grasslands around a paddy field. Our walking route was not constant among days, but it included all habitats in the study site—forests, grasslands and open riverbanks. We spent more than 2000 h conducting this survey.

Another intensive census was conducted for all anuran species from 24 July to 6 November 2012 and from 16 April to 27 July 2013. We walked throughout the study site, mainly during the daytime but occasionally at night, and counted all anurans we encountered. We summed the numbers of each species for each habitat. Our walking route was not constant among days, but it included all habitats in the study site. Metamorphs were excluded from the data because adult *R. tigrinus* generally do not prey on them [22].

(d) Comparison of habitat use

To clarify use of habitat by *R. tigrinus*, we radio-tracked 11 females and 13 males for up to 13 months each between June and November in 2009 and between April and November in 2010. Data for habitat use were obtained from the same individuals as in our previous paper [18], which details the methods for radio telemetry. All the females used for radio telemetry were confirmed to have oviducal eggs by palpation. We tried to locate each individual during the daytime approximately once a week. Habitat was recorded at each location. Intervals between successive locations were more than 3 days, to limit autocorrelation of the data. Toads were found solely in forests (see Results); thus snakes are expected to select forests when they forage for toads. To compare the frequency of forest use between the sexes and to explore the effects of other factors, data were analysed using generalized linear mixed models (GLMMs) with habitat of snake location points as a binary response variable (forest or other habitats). Explanatory variables were sex, season (May–June, July–August, September–October or November–April), snout–vent length (hereafter SVL, measured upon initial capture), weather (fair, cloudy or rainy), and the interaction between season and one of the other variables. Individual identity was used as a random factor. We used the glmmML package [23] in R v. 2.14.2 [24] to fit the models to the data. Binomial errors and a logit link function were used. The model that yielded the lowest Akaike’s information criterion (AIC) was selected from all possible models with or without each variable and interaction.

(e) Behaviour and diet of gravid females

We radio-tracked another group of nine gravid *R. tigrinus* from 16 April to 16 July 2013 to investigate their foraging behaviour and diet. Methods used for snake collection and transmitter implantation were the same as those presented previously [18]. Tracked snakes were located during the daytime once every day except rainy days and except before periodic stomach content examination (see below), and behaviour was recorded. Behaviours of the snakes were categorized as follows: ‘immobile’ indicates snakes remaining in a fixed location, including cases when the snakes were either exposed or concealed; ‘foraging’ indicates snakes pushing their snout towards substrate or poking their head under objects on the ground (e.g. fallen logs) while crawling slowly and flicking the tongue frequently; ‘feeding’ indicates snakes grasping prey with their jaws; and ‘moving’ indicates snakes were crawling without showing obvious behaviour related to foraging or feeding.

We captured the tracked snakes for stomach content examination under two circumstances. First, when a snake showed foraging behaviour at a daily location, we captured it at the first subsequent location where the snake exhibited behaviour other than foraging; this procedure was intended to capture the snakes soon after their foraging bouts. Second, we periodically captured all tracked snakes at approximately two-week intervals except during ecdisis. To ascertain the foraging habitat, we located the snakes three to five times per day during the preceding 3 days (prey remains in the stomach for approx. 3 days [21]) before the periodic examination; behavioural data were not collected for those locations. When the snakes moved between habitats, we avoided periodic stomach content examination for 3 days after such movement. The presence or absence of undigested stomach contents was determined by palpation immediately after capture. If a snake did not have stomach contents, it was immediately released. If it had stomach contents, the snake was force-regurgitated and identified the prey, then force-fed it to the snake in order to return contents to the stomach and released the snake as soon as possible (usually within 1 min of capture). In addition, we recorded prey species when we directly observed tracked snakes consuming prey.
In 2011–2013, we collected 47 (23 females and 24 males) adult R. tigrinus during the gestation season (May to June) and 30 (15 females and 15 males) during the non-gestation season (late July to October). All but one female from the gestation season had ovidual eggs. Data obtained from the single non-gravid female were excluded from statistical analyses. Snakes were brought to a laboratory at the study site and individually housed in cages (32 × 17 × 12 cm) pending experimental trials. Snakes were held without food but were supplied with water ad libitum. When the snakes had stomach contents upon capture, they were tested after completely digesting the prey.

We tested preference for prey trails using a Y-maze. The maze was constructed of clear acrylic pipes and consisted of a base arm (length × diameter: 17 × 7.5 cm) and two diverging arms (18 × 7.5 cm) at a 125° angle to the base arm. One box (47 × 34 × 24 cm) was attached at the start of the maze and two boxes (29 × 21 × 24 cm) at the ends. We prepared chemical cues of the toad (Bufo japonicus formosus) and a non-toxic frog (R. arboreus) by gently scrubbing either of the anurans with a piece of wet laboratory paper (Kimwipe, Kimberly Clark; folded to 20 × 1 cm) with a standardized pressure. We made the trails of the anurans by placing four pieces (two per trail) of the treated paper on the floor of the tubes, extending continuously from the beginning of the base arm to the ends of the divergent arms. On the base arm, the papers from the two anurans were placed side by side, on the same side (respectively) as the scented arm. Assignment of the anurans to the right and left arms was balanced.

Experimental trials were conducted between 09.00 and 16.00. We placed one snake in the starting box, which was separated from the maze by a removable partition. After 20 min acclimation, we removed the partition. Snakes typically proceeded from the base arm into the box on the left or right arm, showing behaviours related to foraging (pushing their snout towards the stimuli, poking their head under the stimuli and/or frequent tongue flicks). We determined a snake’s choice when its snout entered one of the end boxes or when the snake bit the stimulus. After each trial, we removed all paper strips and thoroughly washed the apparatus. Snakes that did not choose either trail within 1 h were returned to their home cage and tested again approximately 7 days later. All snakes were individually marked by ventral scale clipping after the trial to avoid repeated sampling and released at their site of capture.

Rhodophis tigrinus is sexually dimorphic in body size, females being the larger sex [21], and Bufo japonicus is the largest anuran at the study site [25]. Therefore, it is possible that females prefer toads because larger snakes prefer larger prey, rather than through preference for toxic prey. To test the effect of body size, we divided snakes into two groups based on foraging behaviour in daily observation (see Results) or prey preference in the Y-maze experiments and compared SVL between the two groups.

### 3. Results

#### (a) Habitat use by toads and relative abundance of anuran species

We found 41 toads in the study site, all of which were found in forests. Toads were found from April to November, without clear seasonality in the rate of encounters.

The most abundant anurans in forests, grasslands and open riverbanks were Rana tagoi, R. arboreus and Buergeria buergeri, respectively (table 1). Toads were observed much less frequently than R. tagoi (table 1). The occurrence of R. arboreus and R. schlegeli in grasslands was strikingly seasonal: 99% of all encounters occurred from May to June, during their breeding season. Such distinct seasonality was not observed for other frog species.

#### (b) Sexual and seasonal differences in habitat use

We recorded 186 snake locations from 24 individual snakes. Grasslands were frequently used by the snakes throughout the warmer period of the year, despite the small total area of grasslands (approx. 2%). Males predominantly used grasslands from May to June, whereas females, which were gravid in those months, used grasslands and forests at similar frequencies (figure 1). GLMM analysis supported a significant
Table 2. AIC ranking of the models that explain the frequency of forest use. For simplicity, models containing the explanatory variables that did not affect frequency of forest use are not shown. The model that yielded the lowest AIC had ‘season’, ‘sex’ and ‘season × sex’ as explanatory variables. The models were slightly improved by the addition of the explanatory variable ‘sex’ (ΔAIC ≤ 0.3) and were improved to a greater extent by the addition of ‘season × sex’ (ΔAIC ≥ 2.5). This indicates that the overall (i.e. year-round) frequency of forest use differed only slightly between sexes, and the sexual difference was greater in particular season(s). With respect to variables other than ‘sex’, ‘season’ had a relatively large effect on improving the models (ΔAIC ≥ 7.6). The addition of ‘SVL’, ‘weather’, ‘season × SVL’ and ‘season × weather’ increased AIC for the models.

### Table 2

<table>
<thead>
<tr>
<th>ranking</th>
<th>model term(s)</th>
<th>AIC</th>
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<tbody>
<tr>
<td>1</td>
<td>sex, season, sex × season</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>5</td>
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<td>242.4</td>
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</table>

Table 3. Maximum-likelihood estimates of the coefficients for each term in the lowest AIC model. From May to June, females used forests more than males, and this resulted in a negative coefficient value of ‘sex: male’. Such an obvious sexual difference in forest use was absent during other seasons. The frequency of males using forest in May–June tended to be lower than in other seasons, and this resulted in positive coefficient values of ‘sex: male × season: Jul–Aug’, ‘sex: male × season: Sep–Oct’ and ‘sex: male × season: Nov–Apr’.

### Table 3

| model term | estimate | s.e. | Pr(>|z|) |
|------------|----------|------|----------|
| intercept  | −0.67    | 0.43 | 0.11     |
| sex: male  | −2.27    | 0.89 | 0.010    |
| season: Jul–Aug | 0.00 | 0.57 | 1.0     |
| season: Sep–Oct | 0.40 | 0.66 | 0.54    |
| season: Nov–Apr | 1.41 | 0.69 | 0.041   |
| sex: male × season: Jul–Aug | 2.80 | 1.05 | 0.0074  |
| sex: male × season: Sep–Oct | 1.51 | 1.15 | 0.19    |
| sex: male × season: Nov–Apr | 1.37 | 1.16 | 0.24    |

Table 4. Comparison of diet composition of gravid females among foraging habitats.

### Table 4

<table>
<thead>
<tr>
<th>foraging habitat</th>
<th>prey species</th>
<th>Bufo japonicus (toad)</th>
<th>Rhacophorus spp.</th>
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</thead>
<tbody>
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<td>forest</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>grassland</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
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</table>

Five of the nine gravid females monitored daily were located in forests for 13–24% of observations (overall, 32 of 184 locations occurred in forest) and exhibited foraging behaviour in that habitat. In some cases (n = 7), they travelled a round trip between an open habitat (grassland or open riverbank) and forest, with 1- to 3-day stays in forest. By contrast, the remaining four gravid females were seldom (only once) or never located in forest (two of 185 locations occurred in forest) and never showed foraging behaviour in that habitat. The former snakes mainly fed on toads, whereas the latter snakes fed mainly on Rhacophorus and were never found to have eaten toads (table 5).

(d) Sexual and seasonal differences in preference for prey trails

During the gestation season, the majority of gravid females preferred the trail of a toad, although this bias fell short of statistical significance (binomial test, hereafter BT, \( p = 0.052 \); figure 3). Males significantly preferred the non-toxic frog R. arboreus (BT, \( p = 0.023 \)). These proportions were significantly different between sexes (Fisher’s exact test, hereafter FET, \( p = 0.0027 \)). One female that did not have oviducal eggs chose R. arboreus.

During the non-gestation season, females significantly preferred R. arboreus (BT, \( p = 0.0074 \)). Two-thirds of males chose R. arboreus, although the preference was not statistically significant (BT, \( p = 0.30 \)). There was no significant sexual difference in prey preference during the non-gestation season (FET, \( p = 0.39 \)).
Prey preference of females significantly differed between the gestation and non-gestation seasons (FET, $p = 0.0063$), whereas males did not show such seasonal change (FET, $p = 0.72$).

(e) Effects of body size on foraging behaviour and prey preference

There was no significant difference in SVL between the gravid snakes that foraged in forests (mean ± s.d., 841 ± 35 mm) and those that did not (864 ± 89 mm; Wilcoxon rank sum test, $p = 0.29$).

There was no significant difference in SVL between females that chose the trail of the toad (795 ± 80 mm) and those that chose the trail of $R$. arboreus (840 ± 108 mm; Wilcoxon rank sum test, $p = 0.12$), nor between males that chose the toad (637 ± 34 mm) and those that chose $R$. arboreus (627 ± 63 mm; Wilcoxon rank sum test, $p = 0.84$).

4. Discussion

Our census survey of anurans showed that toads are much less abundant than some other anurans, implying that snakes that forage for toads incur potential costs by increased foraging effort (e.g. energetic costs, exposure to predators [26,27]). Unsurprisingly, a previous survey of stomach contents reported that toads are an infrequent food for $R$. tigrinus at this study site [20]. Nonetheless, our daily observations and stomach content data revealed that some gravid $R$. tigrinus mainly feed on toads by actively foraging in the habitat of toads. Selective foraging while in the forest is strongly suggested because $R$. tagoi, a typical prey of $R$. tigrinus [19,28], was not found in stomach contents despite its much higher density than toads. Our Y-maze experiments demonstrated that gravid females are more likely to choose the trail of toads than do males or non-gravid females. These results imply that there might be some physiological or neural mechanism regulating responses to chemical cues of prey. Our data showed no effects of body size on prey preference, and non-gravid females prefer $R$. arboreus to toads. These results negate the possibility that females prefer toads because larger snakes prefer larger prey. Overall, our results support the hypothesis that gravid females actively acquire defensive chemicals for provisioning their offspring.

Although females used forests more frequently than did males during the gestation season, that sexual difference in habitat use was due to decrease of forest use in males, rather than increase of forest use in females while gravid. Why did males tend to avoid forests and predominantly use grasslands from May to June? Two factors may contribute to preference for grasslands. First, grasslands offer simultaneous access to suitable basking sites and shade from grass clumps. Such habitat offers a thermal gradient that facilitates thermoregulation and thus is often preferred by snakes [29–31]. Second, abundant food in grasslands would attract snakes between May and June. In that season, $R$. arboreus and $R$. schlegelii, the main foods of $R$. tigrinus at AFRS [20], aggregate in grasslands for reproduction in large numbers: we counted more than 500 foam nests of $R$. arboreus at one time in roughly 0.3 ha of paddy field. Thermoregulation and food acquisition affect habitat use by snakes [31–33], so it is not surprising that male snakes prefer grasslands from May to June.

Why did female $R$. tigrinus shuttle between grasslands and forests in the gravid season of May and June? In general, gravid female snakes prefer open habitats more than do males or non-gravid females [34–36], presumably for precise thermoregulation, which assists in embryonic development [37–39]. Use of forests by gravid $R$. tigrinus is opposite to the typical pattern. There are at least two possible explanations for the combined use of grasslands and forests by gravid $R$. tigrinus. (i) The pattern may arise from a balance between thermoregulation and toad-eating; use of grasslands as ‘home area’ and use of forests for intermittent foraging for toads ($R$. tigrinus feeds every several days [21]). Our data are consistent with this possibility; foraging behaviour was rare in grasslands but common in forests, and gravid females fed mainly on toads while in forests. (ii) Gravid females may prefer forests because fewer predators occur there. However, even if predators are uncommon in forests, shuttling between two habitats would be ineffective for avoiding predation because long-range movements presumably increase exposure to predators. If the snakes used forests as a retreat, they would be expected to remain there longer, which was not the case. Moreover, predator avoidance
cannot explain why gravid females actively foraged in forests and ate toads rather than more abundant prey. Therefore, exploitation of toads, rather than predator avoidance, is a more likely explanation for use of forests by gravid females.

In *R. tigrinus*, maternal provisioning of bufadienolides appears to be an essential mechanism to arm hatchlings for more than half a year after hatching. Because of gape limitation, neonate snakes, which hatch in late summer, are barely capable of exploiting toads on their own until the following spring, when metamorphosed toadlets become available [40]. Given the vulnerability of juvenile snakes to predation [41], the presence of defensive toxins in the nuchal glands is expected to be especially important in juveniles. As exploitation of toads by gravid females results in hatchlings that are more richly endowed with defensive compounds [6], maternal foraging for the rare, toxic prey presumably contributes to offspring survival by enhancing chemical defense in the vulnerable life stages (i.e. juvenile).

An unexpected finding is the variation in foraging and diet among individual gravid females of *R. tigrinus*. We do not know whether the observed foraging pattern was directly reflected in the defensive level of hatchlings; we cannot dismiss the possibility that some gravid females had already obtained and provisioned toxins before our monitoring. Alternatively, it is possible that some females do not forage for toads, producing offspring with little or no toxins. Juvenile *R. tigrinus* possess a bright yellow collar, suggesting aposematism. Therefore, toxin-free juveniles might receive some protection from predators by displaying ‘dishonest’ warning signals. Coexistence of well-defended and less-defended individuals in an aposematic species is called automimicry (after [42]), and its evolutionary persistence receives strong theoretical support [43,44]. Obviously, further chemical and behavioural investigations are required to demonstrate whether *R. tigrinus* exhibits automimicry. Insights into the factors underlying differences in foraging behaviour are a challenge for future studies.

In summary, our results demonstrated behavioural switching in females, associated with active foraging for scarce, toxic prey during gestation. Coupled with data from previous studies, it is likely that active exploitation of toads by gravid females is associated with defensive provisioning of their offspring. This study provides a unique vertebrate example of a complex system in which offspring protection results from a seasonal shift in parental foraging behaviour.

Data accessibility. Data are available in Dryad (doi:10.5061/dryad.s0h82).

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