ELSEVIER

Physiology & Behavior 75 (2002) 237–241

PHYSIOLOGY & BEHAVIOR

Lingual and biting responses to selected lipids by the lizard *Podarcis lilfordi*

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Received 9 August 2001; received in revised form 25 September 2001; accepted 31 October 2001

Abstract

Many lizards can identify food using chemical cues, but very little is known about the chemical constituents used for this purpose. We experimentally investigated responses to several lipid stimuli by the omnivorous lacertid lizard *Podarcis lilfordi*, which had been shown previously to be capable of identifying prey using only chemical cues and to respond to pork fat by tongue-flicking and biting. In 60-s trials in which stimuli were presented on cotton swabs, the lizards responded very strongly to pure pork fat and to oleic acid, but not to cholesterol or glycerol. Latency to bite swabs, the number of individuals that bit swabs, and the tongue-flick attack score, TFAS(R), which combines effects of tongue-flicks and bites, showed stronger responses to fat than to cholesterol, glycerol, and distilled water but did not differ significantly from responses to oleic acid. Several lines of evidence show that oleic acid elicited strong chemosensory and feeding responses. For individuals that did not bite, the number of tongue-flicks was significantly greater for oleic acid than for distilled water or glycerol, and nearly so for cholesterol. Latency to bite was significantly shorter for oleic acid than for distilled water, and TFAS(R) was significantly greater for oleic acid than for distilled water and glycerol. In combination with pilot data indicating no strong response to the waxy, saturated palmitic acid, these findings suggest that oleic acid in particular and probably other unsaturated fatty acids found in animal fat contribute strongly to the food-related responses to lipids. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Behavior; Lipids; Food discriminations; Chemical senses; Squamata; Lacertidae; *Podarcis lilfordi*

1. Introduction

Squamate reptiles can identify food by tongue-flicking, but little is known about the types of chemicals that are important in this regard beyond the finding that garter snakes of the genus *Thamnophis* respond to specific proteins from earthworms [1,2]. Among lizards, active foragers and omnivorous or herbivorous species can identify and evaluate foods using chemical cues sampled by tongue-flicking (e.g., Refs. [3–6]). In contrast to the extensive information now available about the relationships between foraging mode and prey chemical discrimination [3,7] and between plant diet and plant chemical discrimination [6,8], very little is known for lizards about the kinds of chemicals that stimulate increased tongue-flicking rates and permit identification of food as indicated by feeding attempts.

In the first study of responses to major categories of organic compounds found in foods by lizards, strongest lingual and biting responses by the lacertid *Gallotia caesaris* were detected for the carbohydrate sucrose and for fat, which contains a variety of lipids [9]. A second study revealed similar behavior by the lacertid *Podarcis lilfordi*, which additionally exhibited a stronger response to protein than to deionized water and a different sort of response to protein than to sucrose, which elicits licking [10]. The response to protein was much weaker than to fat or sucrose. This contrasts with findings for *Thamnophis*, in which specific proteins from earthworms, a favored food, elicit very strong responses [1,2]. The lack of strong response to protein by *P. lilfordi* may be artifactual because the protein tested was bovine gamma globulin rather than protein from the integument or exoskeleton of typical prey. Additional studies are needed to ascer-

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PII: S0031-9384(01)00656-4

tain the types of lipids, carbohydrates, and proteins that may contribute to chemical discriminations regarding prey and plant foods by lizards.

Here, we report a first examination of the effects of a few categories of lipids on chemosensory sampling and feeding behavior. Both *G. caesaris* and *P. lilfordi* responded very strongly to pure pork fat, as indicated by biting attacks following chemical sampling by tongue-flicking [9,10]. Because fat is composed primarily of trigylcerides, triglycerides and their constituent molecules are the most likely candidates as the effective compounds. The two major categories of fatty acids, saturated and unsaturated, seem likely to have different effects because at ambient temperatures unsaturated fatty acids may be accessible as liquids, whereas saturated fatty acids may be waxes. Glycerol, the other major constituent of triglycerides, is potentially important because it is ubiquitous. Other categories of lipids, such as sphingolipids, might also elicit strong responses whether or not they are abundant in fat.

We experimentally studied responses to several lipids presented on cotton swabs to *P. lilfordi*, an actively foraging omnivore. This species was used because it is known to be capable of prey chemical discrimination and to respond strongly to fat [10,11] and is very abundant. Pork fat was used as a stimulus to estimate the strength of the full response to a natural mixture of lipids, and water was used as an odorless control to determine response level in the absence of any stimulus related to food. In addition, comparisons of responses to a few readily available lipids (i.e., a single representative each of saturated and unsaturated fatty acids, glycerol, and cholesterol) allowed us to determine whether each type of lipid caused any increase in lingual and biting responses and the response strength relative to the full response to fat.

2. Methods

Twenty adult male *P. lilfordi* were collected by noosing and with traps baited with fruit on Aire, an islet offshore from Menorca, Balearic Islands, Spain. The lizards were transported to a laboratory on Menorca, where they were housed individually in transparent 40.5 × 25.0 × 26.5 cm plastic terraria. Each terrarium contained a floor of indoor–outdoor carpet and a water dish, and all sides were covered with white paper to reduce disturbance to the lizards due to movement by the experimenters in the room. The natural photothermal cycle was supplemented by heat lamps, one at an end of each cage, which raised air temperature in cages to 30–32 °C during testing and permitted thermoregulation. Lizards were left in cages overnight on the day of capture and were tested on the following two days.

Lipid stimuli were presented to lizards on the cotton tips of 15-cm wooden applicators. Deionized water served as an odorless control to assess response to the experimental milieu in the absence of stimuli relevant to food. Pure pork fat was tested as a stimulus containing several types of lipids. Oleic acid was used as an example of a nonsaturated fatty acid, glycerol as a component of triglycerides, and cholesterol as an additional lipid. After pilot tests revealed no strong response to the waxy palmitic acid, this saturated fatty acid was not included in the experiment. Swabs were impregnated with stimuli as follows: Distilled water, glycerol, and oleic acid stimuli were prepared by immersing the swab in the corresponding liquid; cholesterol stimuli were prepared by immersing the swab in 5 ml of a solution of 0.025 g of cholesterol in glycerol. Pure pork fat stimuli were prepared by inserting a swab into fat at room temperature and wiping off excess with a paper towel. To human observers, the swabs prepared with all stimuli were similar.

Each trial was initiated by slowly moving a cotton swab to a position 1.0–1.5 cm anterior to a lizard's snout. Starting with the first tongue-flick directed to the swab, the experimenter recorded the number of tongue-flicks directed to the swab in 60 s if the lizard did not bite. If the lizard bit, the latency to bite in seconds from the first tongue-flick until the bite was recorded, as well as the number of tongue-flicks prior to the bite.

The experiment was conducted on 15–16 May 2001 between 11:15 and 18:30 h. All data were collected by a single experimenter. Each lizard was tested with all stimuli in a repeated-measures (randomized blocks) design with a minimum intertrial interval of 30 min. To prevent possible bias that might occur if all lizards were tested using the same sequence of stimuli, the stimulus sequence was varied among individuals by incomplete counterbalancing in which one of the sequences starting with each stimulus type was randomly eliminated.

Variables examined statistically were the number of tongue-flicks, latency to bite, proportion of individuals that bit, and TFAS(R), the tongue-flick attack score for repeated measures [12]. The tongue-flick attack score gives the best overall indication of response strength by combining tongue-flicks, which reflect chemosensory investigation, with biting, which shows predatory attack [12–14]. In trials without bites, TFAS(R) is the number of tongue-flicks. In trials with bites, TFAS(R) is the maximum number of tongue-flicks in any trial by the same individual plus 60 minus the latency to bite in seconds. Thus, a bite is given heavier weight than any number of tongue-flicks.

We initially planned to analyze data on tongue-flicks, latency to attack, and TFAS(R) using analysis of variance for a single factor experiment having a randomized blocks design [15]. Because variances of all of these variables were significantly heterogeneous as indicated by Hartley's Fmax tests, both for raw and logarithmically transformed data, we instead conducted nonparametric Friedman two-way analyses of variance [16]. When significant main effects were found, a procedure described in Ref. [16] for nonparametric paired comparisons was used to test the significance of differences between pairs of stimulus means.

The main effect of stimulus condition on the proportion of individuals that bit was assessed using a Cochran *Q* test followed by sign tests of the differences between pairs of conditions [16]. Raw probabilities of the binomial tests are reported, but a sequential Bonferroni procedure was used to evaluate significance [17]. All significance tests were two-tailed, with $\alpha = .05$. Two lizards failed to complete the experiment, one ceasing responses to swabs after two trials, the other after four. Thus, the sample size for all statistical analyses was 18.

3. Results

Numbers of tongue-flicks were highly variable, especially in the oleic acid condition, which had the highest mean values (Table 1). The lowest number of tongue-flicks occurred in the fat condition, but only because almost all individuals bit too quickly to permit many tongue-flicks. There were no significant differences among conditions in the number of tongue-flicks ($\chi^2 = 6.10$, $df = 4$, $P > .10$).

When the data are limited to trials in which bites did not occur, oleic acid elicited much greater numbers of tongue-flicks than cholesterol, glycerol, and water (Table 1). Eight individuals did not bite in any of the oleic acid, glycerol, and water conditions, permitting sign tests. All eight individuals had higher numbers of tongue-flicks in the oleic acid condition than in either of the others. Numbers of tongue-flicks were significantly greater in response to oleic acid than to glycerol or water (sign test, $P < .0079$ each) when no bite occurred. Using a Mann–Whitney *U* test to compare tongue-flicks between oleic acid and cholesterol when no bite occurred, there were six individuals that bit in the oleic acid condition, but not the cholesterol condition, and four individuals that bit in the cholesterol condition, but not in the oleic acid condition. For these individuals, the difference is not significant, but is marginal despite the small sample size ($U = 5.0$; $n = 4$, 6, $P = .086$), with greater tongue-flicks in the oleic acid condition.

Similar comparisons among other pairs of stimuli in trials without bites were not significant, consistent with similarity of their means (Table 1). In the comparison between eight lizards that bit in neither the cholesterol nor the glycerol condition, the number of tongue-flicks was greater to cholesterol for four individuals and for glycerol for the other four ($P > 0.10$). There was no difference between numbers of tongue-flicks in the glycerol and water conditions when bites did not occur (nine individuals, cholesterol > water in six cases, water > cholesterol in three cases, $P > 0.10$). Among 12 individuals that bit in neither the glycerol nor the distilled water condition, the number of tongue-flicks was greater in response to glycerol for seven lizards, greater to water for four lizards, and there was one tie. This difference was not significant ($P > 0.10$).

Table 1
Mean tongue-flicks by *P. lilfordi* responding to chemical cues from fat (FAT), oleic acid (OLE), cholesterol (CHO), glycerol (GLY), and distilled water (WAT) on cotton swabs in 60-s trials

	FAT	OLE	CHO	GLY	WAT
All trials ($n = 18$)					
Mean	4.56	16.67	9.17	8.00	7.11
S.E.	1.07	3.91	2.36	1.89	1.58
Range	1–17	1–52	1–39	1–30	1–25
Trials without bites					
n	1	8	10	12	14
Mean	15.00	31.25	13.00	9.58	7.00
S.E.	–	4.79	3.69	2.55	1.84
Range	–	14–52	2–39	2–30	1–25

Table 2
Biting responses by 18 *P. lilfordi* responding to chemical cues from fat (FAT), oleic acid (OLE), cholesterol (CHO), glycerol (GLY), and distilled water (WAT) on cotton swabs in 60-s trials

	FAT	OLE	CHO	GLY	WAT
Latency to bite (s)					
Mean	8.72	29.00	36.39	42.39	49.39
S.E.	3.46	6.77	6.56	6.14	5.01
Range	1–60	1–60	1–60	1–60	2–60
Individuals that bit	17	10	8	6	4

Mean latency to bite varied greatly among conditions, being far shorter in the fat condition than in all others and somewhat shorter in the oleic acid condition than in the remaining conditions (Table 2). The main stimulus effect was highly significant ($\chi^2 = 30.55$, $df = 4$, $P < 1.0 \times 10^{-5}$). Paired comparisons showed that latency to bite was significantly shorter in response to fat than to cholesterol ($P < .05$), glycerol ($P < .005$), and distilled water ($P < .001$). Despite the substantially shorter mean latency to bite swabs bearing fat than oleic acid, this difference was not significant ($P > 0.10$). The only other significant difference between pairs of conditions was the shorter latency to bite in the oleic acid condition than in the distilled water condition ($P < .05$).

Numbers of individuals that bit (Table 2) exhibited a similar pattern to latency to bite. The stimulus effect was highly significant ($\chi^2 = 26.32$, $df = 4$, $P < .001$). A significantly greater proportion of lizards bit in the fat condition than in the cholesterol ($P = .002$), glycerol ($P < .001$), and distilled water ($P < .00025$). The only other substantial differences were those between fat and oleic acid ($P < .016$) and between oleic acid and water ($P < .032$). The latter two differences approached significance, but were not significant at the adjusted Bonferroni levels of .0072 and .0083, respectively, for the 10 possible comparisons.

TFAS(R) (Fig. 1) exhibited the same trends as the two variables based solely on bites, but permitted slightly greater resolution of differences among stimuli. The ranges of TFAS(R) were 15–110 for fat, 14–97 for oleic acid, 2–110 for cholesterol, 2–77 for glycerol, and 1–64 for water. Differences among stimuli were highly significant ($\chi^2 = 37.77$, $df = 4$, $P < 1 \times 10^{-5}$). Mean TFAS(R) was significantly greater in response to fat than to cholesterol ($P < .05$), glycerol ($P < .005$), and distilled water ($P < .001$). TFAS(R)

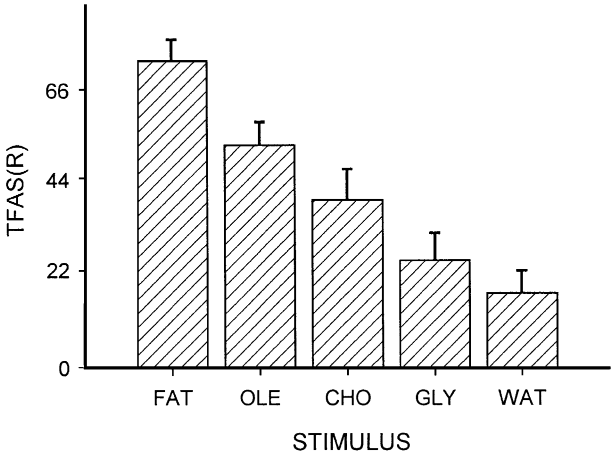

Fig. 1. Mean tongue-flick attack scores for 18 adult male *P. lilfordi* in 60-s trials responding to cotton swabs bearing pure pork fat (FAT), oleic acid (OLE), cholesterol (CHO), glycerol (GLY), and distilled water (WAT). Error bars represent 1.0 S.E.

was also significantly greater in the oleic acid condition than in the glycerol ($P<.02$) and distilled water conditions ($P<.001$). No other differences were significant although those between cholesterol and distilled water and between oleic acid and glycerol were substantial.

4. Discussion

Lipids are a major stimulus contributing to prey chemical discriminations by the two species of lizards in which their effectiveness has been examined and presumably in other lizards. As in previous studies of lacertid lizards [9,10], pure pork fat elicited very strong responses from *P. lilfordi*. Although the number of tongue-flicks was slightly lower in the fat condition than in the other conditions, this difference was not significant. When responding to fat stimuli, most of the lizards simply bit too quickly to permit many tongue-flicks. The high percentage of individuals that bit and the short latency to bite indicate that fat stimuli were rapidly identified as food.

Oleic acid elicited very strong lingual and biting responses. This is shown by the significantly greater TFAS(R) to oleic acid than to glycerol and distilled water and the significantly shorter latency to bite swabs bearing oleic acid than distilled water. Although there were no significant differences among conditions in the number of tongue-flicks, the mean number of tongue-flicks to oleic acid was more than twice that for either glycerol or distilled water. For TFAS(R), response strength to oleic acid was 2.95 times that to distilled water. These strong responses to oleic acid suggest that this abundant constituent of mammalian fat may be an important component of pork fat for its identification as a food.

Although the mean response strength to oleic acid was statistically indistinguishable from that to pork fat, it was quantitatively only 0.73 of that to fat for TFAS(R). This suggests that additional components of fat may contribute to the overall response because even pure oleic acid, a supernormal stimulus probably never encountered naturally, appears to be slightly less effective than a natural lipid mixture. The ability of other triglycerides found in fat, such as palmitoleic, linoleic, linolenic, and arachidonic acids, to induce tongue-flicking and biting should be studied.

Cholesterol and glycerol had no significant effect on any of the variables analyzed. The number of tongue-flicks for these stimuli was very similar to that for distilled water. Although the difference was not significant, slightly more individuals bit and at shorter latency in response to cholesterol in glycerol than to distilled water, suggesting that cholesterol or its combination with glycerol might have a small effect requiring a larger sample size or a higher concentration for its demonstration. Further hints of such an effect are that cholesterol in glycerol elicited a response 2.23 times that to distilled water for TFAS(R) and that the strength of response to the solution of cholesterol in glycerol was slightly greater than half of that to fat, 0.55 for TFAS(R). However, no effect was demonstrated, and the lack of effect agrees with the finding that the cholesterol derivative estradiol-17β in peanut oil did not elicit a higher rate of tongue-flicking by male insectivorous lizards, *Eumeces laticeps*, than did peanut oil alone [18].

Tongue-flicking, which serves to sample chemicals for analysis by vomerolfaction, and presumably stimulates gustatory responses as well, is a convenient empirically observable indicator of chemosensory investigation by lizards. It is also possible that the stimuli had different odors detected by the lizards via olfaction, which can activate tongue-flicking for vomerolfactory analysis [19]. Biting provides an indication of predatory attack that in most actively foraging, insectivorous lizards occurs exclusively or nearly so as a response to prey chemicals (e.g., Refs. [20–22]). Such bites can readily be distinguished from defensive bites, which are not usually preceded by tongue-flicks in *P. lilfordi*, are accompanied by defensive postures, are brief, and are typically followed by retreat of the lizard. Defensive tongue-flicking may occur in some snakes, but is rare or absent in lizards [23]. *P. lilfordi* is unusual among lizards in that it bites more frequently in response to control stimuli such as water than do most lizards. This might be a consequence of intense competition for food in the very dense populations on Aire [11] or of the omnivorous diet of this species.

Tongue-flicks and bites are by far the most useful variables for the study of prey chemical discriminations. In addition, a shift in body position, especially elevation and/or turning of the head, often accompanied by locomotory movement for a very short distance, sometimes occurs immediately following tongue-flicks contacting a swab. Such behavior has been observed at low frequency in many lizard species (Cooper, unpublished). We observed such behavior in three individuals, one each in the oleic acid, cholesterol, and glycerol conditions. The behaviors hint that detection of chemicals, indicating the possible presence of food, may induce visual search for a prey item. In trials with presumptive search behavior, the greatest numbers of tongue-flicks in any trial were observed for glycerol (30) and cholesterol (39), and the third greatest for oleic acid, indicating intense chemosensory investigation. For other individuals, prey chemicals on a small white object, the swab, may suffice to release predatory attack or further tongue-flicks.

The importance of lipids to chemosensory identification of food by lizards seems clear, but the types of lipids that are most important in this regard remain largely unknown. The present findings show that one specific unsaturated fatty acid elicits very strong responses, ones that could not be distinguished statistically from that to a mixture of lipids in fat. In addition, they hint that unsaturated fatty acids may be among the primary effective compounds. However, the only saturated fatty acid tested was palmitic acid, which had no detectable effect on tongue-flicking and did not elicit bites but is not a major constituent of animal fat [24].

Adult *P. lilfordi* consume a wide variety of prey and plant foods [11] that may contain a wide range of lipids. Oleic and other common fatty acids may be present in a wide range of food species. Similarities among them, especially among unsaturated fatty acids, might permit identification of a wide range of food species, even foods previously unencountered. Future studies should examine responses to additional animal fatty acids and to a range of lipids as yet untested, as well as to mixtures of lipid types. Although lipids may be adequate to induce predatory attacks, their roles with respect to other classes of organic chemicals remain unknown. Studies of the effects of combinations of proteins, lipids, and carbohydrates found in preferred prey on lingually mediated chemosensory discriminations are needed to detect any synergisms or additive relationships.

Acknowledgments

This study was partially supported by project PB98-0270 of the Spanish Ministry of Education and Culture, by the Institut Menorqui d'Estudis, Consell Insular de Menorca, and by an international travel grant from Indiana University-Purdue University. Lizards were collected under a permit to VPM from the Servei de Biodiversitat, Conselleris d Medi Ambient del Govern.

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