Rapid onset of mate-quality assessment via chemical signals in a woodland salamander (*Plethodon cinereus*)

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Abstract

In the competition to acquire mates, environmental factors can be important in determining the relative quality of an individual. These aspects of quality are often conveyed through signals used for mate assessment by the most energetically-invested sex. In red-backed salamanders, *Plethodon cinereus*, chemical signals communicate a surprising amount of information, much of which is dependent on the current condition of the sender. One such condition-dependent aspect of information conveyed via chemicals is the quality of the sender's diet, but it is unclear as to whether this information is actively advertised by the sender (i.e., a signal) or simply inferred from food-derived odors (i.e., a cue). The amount of time on different diets required for changes in signaling is also unknown. I examined how quickly gravid female salamanders could detect a difference between the scents of males on high vs. low-quality diets without fecal cues. The amount of protein present in two known signaling glands (the mental and postcloacal glands) was also measured after experimental feeding. Gravid females were able to infer the differences in male diet quality after only one week. Females also responded to the male scents more quickly after differential feeding had begun. High-quality males had significantly more protein present in both signaling glands than low-quality males. This scenario highlights the ongoing interplay between the quality of an individual and its environment, with males actually advertising the status of this relationship as an honest signal for mate assessment.

Key words:

Mate assessment, Condition-dependent signal, Diet

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Introduction

Current evolutionary theory has grown to include the profound effects that environmental factors can have on the development of phenotypes and the reproductive success of individuals (Jablonka and Lamb 1995; West-Eberhard 2003). One of the most significant environmental factors that can affect fitness is the availability and procurement of dietary resources. Energetic and nutritional resources do not solely mediate pressures due to natural selection (e.g., starvation); they can have implications in terms of sexual selection. Females of many species prefer males of higher "quality," as assessed by signals conveying information about their diets (Walls et al. 1989; Ferkin et al. 1997; Fisher and Rosenthal 2006). Other condition-dependent information may also be utilized by females to assess potential mates, such as male health and parasite-load (Penn and Potts 1998; Maksimowich and Mathis 2001; Doucet and Montgomerie 2003) or dominance status (Moore et al. 1997; Kortet and Hedrick 2005). In general, condition-dependent signals serve to provide females with information regarding behavioral, physiological and even potentially genetic information that indicates the sender as a potentially valuable reproductive partner (Andersson 1994).

Though examples of mate quality indicators in the realm of audiovisual signals are prevalent across numerous taxa (Davies and Halliday 1978; Clutton-Brock et al. 1979, 1982; Kodric-Brown and Brown 1984; Welch et al. 1998), chemical signals can also be used to assess male condition. For example, females are known to prefer the scents of males with higher dietary protein content in swordtails, *Xiphophorus birchmanni* (Fisher and Rosenthal 2006), and meadow voles, *Microtus pennsylvanicus* (Ferkin et al. 1997). If individuals communicate information about their relative quality, as partially dictated by environmental factors, the question then arises as to the actual mechanism by which the environment can modulate communication between organisms. Another question of interest is how quickly the effects of an altered environmental factor can be manifested in communication. The chemical signaling system of plethodontid salamanders is an excellent model in which to investigate these questions.

The communication of plethodontid salamanders relies heavily upon non-volatile chemicals deposited on the substrate in the form of fecal pellets or glandular markings (Jaeger 1986; Simons et al. 1994, 1997, 1999), volatile compounds (Martin et al. 2005; Dantzer and Jaeger 2007a, b), as well as molecules transferred directly from the sender to the receiver (Rollmann et al. 1999). These chemical signals facilitate a wide variety of inter- and intraspecific functions, such as prey detection (Dickens 1999; Placyk and Graves 2002), predator-avoidance (Murray and Jenkins 1998; Maerz et al. 2001; Sullivan et al. 2005), territoriality (Jaeger 1986; Quinn and Graves 1999), homing and orientation (Madison 1969; Madison and Shoop 1970; Kleeberger and Werner 1982), as well as mate choice and courtship (Rollmann et al. 1999; Palmer et al. 2007). Plethodontid pheromones also convey a large amount of information about the sender, such as species (Jaeger and Gergits 1979; Dawley 1984, 1986), sex (Dawley 1984; Jaeger et al. 1986; Dantzer and Jaeger 2007a), size (Mathis 1990a; Marco et al. 1998) and individual identity (Madison 1975; Tristram 1977; McGavin 1978; Jaeger 1981; Simon and Madison 1984; Jaeger et al. 1986). Other information transferred by chemical signals relates to condition-dependent aspects of the sender that can change over time, such as the reproductive status of females (gravid vs. non-gravid; Marco et al. 1998; Dantzer and Jaeger 2007b), parasite-load (Maksimowich and Mathis 2001), tail autotomy (partial loss of tail) and injury (Sullivan et al. 2003; Wise et al. 2004), as well as the quality of an individual's diet (Walls et al. 1989).

Female red-backed salamanders (*Plethodon cinereus*) are known to detect the scents of males fed different diets (Walls et al. 1989; Jaeger and Wise 1991), but the exact source of the signals involved has not been examined. . Previous experiments used whole fecal pellets for experimental male odors, and it is therefore unclear as to whether or not the chemicals responsible for conveying this information are actively produced by the male for that function, i.e., a true signal (as opposed to a cue). Here, a cue is defined as any other communication that can convey information or elicit a reaction in the receiver, but was not advertised on behalf of the sender specifically for that purpose (sensu Maynard Smith and Harper 2003). For example, a more parsimonious explanation could be that females infer the quality of the diet from cues in the pellets themselves, such as the proportion of chitinous components (visual cues), or partially undigested food particles (visual/gustatory cues). Indeed, females engage in a "fecal squashing" behavior that might aid in this investigation (Jaeger and Wise 1991; also see Karuzas et al. 2004). Given that females can infer differences between male characteristics using olfaction, then the variation among male characteristics must be encoded within the biochemical composition of the chemical mixtures produced by the males. Two lingering questions remain: (1) what is the biochemical mechanism for this complex information transfer, and (2) how quickly can changes in the environment influence the composition of male chemical signals?

The objective of this research was therefore twofold. First, preliminary experiments were designed to investigate whether females could infer the scents of conspecifics through signals independent of fecal pellets, and second, whether these signals would be capable of conveying information about the quality of the sender's diet. This approach begins to address whether or not the communication of diet-quality is a true signal due to a substance actively produced by cells in the body of the sender. To test this, male odors were obtained by "body washes" to eliminate the confounding factor of fecal pellets and other cues from the male's home substrate.

If the information of diet-quality is actively conveyed by chemicals produced by the sender, then it is predicted that greater energy levels obtained by the sender would translate into an increased investment in signaling. This type of relationship between energy and signal production would therefore be considered an honest signal of mate quality. Chemical signals are considered an excellent mechanism for honest signals because there is a direct energetic requirement for their production (Bradbury and Vehrencamp 1998). To assess whether increased energy leads to greater signal production, I tested males on different quality diets for the levels of protein present in two gland regions known to be important for chemical signaling: the mental gland and postcloacal gland. I predicted that males on high-quality diets will possess greater amounts of secretory protein in the two signaling glands assayed.

The mental gland of plethodontid salamanders is a sexually dimorphic gland found on the underside of the chin (the mentum) of males and is utilized during courtship (Sever 1976; Simons and Felgenhauer 1992). The proteinaceous pheromones from this gland are administered directly to either the snout or the integument of females (depending on the species) and decrease the duration of courtship in multiple species (Houck and Reagan 1990; Rollmann et al. 1999). The postcloacal gland, however, has been implicated in territorial scent marking through a characteristic behavior termed the "postcloacal press" (Simons and Felgenhauer 1992; Simons et al. 1994, 1997, 1999). The glandular secretions of the postcloacal gland are composed mostly of proteins and carbohydrates (Hecker et al. 2003; Chouinard 2010) and these molecules are likely to be responsible for a large amount of the information transferred to receivers via scent marks. Testing the influence of dietary resources on the protein levels of these two known signaling glands will provide additional insight into the source of the signal involved in conveying information about diet-quality and energetic condition. While there are other types of molecules produced by these glands, the level of protein will be used as a proxy for the overall production of signaling glands.

The second objective of this research was to identify the amount of time in which the environment can modulate chemical signaling. To address this question, mate choice by gravid females was used to assess how quickly changes in diet can be detected through chemical signals. I predicted that females will make strong behavioral preferences to associate with high-quality signals as a result of the physiological and behavioral benefits of mating with high-quality males. No a priori predictions were made regarding how long it would take for differences in diet-quality to be inferred by female receivers.

Methods

Testing the Time-course of Female Diet-quality Detection

Animal Collection and Pairing

Salamanders were collected from multiple 20 m^2 plots in Kingman Farm, Durham, NH in May 2008. This time of year is the peak of the spring breeding season in this species, as well as the peak in hypertrophy of chemosensory epithelium (Sayler 1966; Dawley et al. 2000, 2006). Animals were housed individually in the lab in clear plastic containers (15 x 15 x 5 cm) with moist paper towels as substrate. Lab conditions were maintained at 20°C with a 14:10 (L:D) light cycle. The sex of each animal was determined by candling (Gillette and Peterson 2001). Males were identified based on the presence of testes, while gravid females were identified based on the presence of mature ova (after Sayler 1966). Only gravid females were utilized in this experiment. All individuals were also measured for snout-vent length (SVL; mm), total length (TL; mm) and mass (g). Males were arranged into twelve size-matched pairs (± 2 mm SVL; 5 mm TL; 0.1 g). Each of twelve females was assigned to a different male pair, and every female was tested between the scents of the same male pair for the duration of the experiment. As all animals came from multiple plots throughout the collecting site, females were matched to male pairs so that they were never from the same plot as the experimental males. This precaution was necessary considering evidence for chemical signals conveying aspects of spatial information and familiarity (Madison 1975; McGavin 1978; Jaeger 1981). Aside from this stipulation, females were matched randomly to male pairs.

Experimental Outline and Male Feeding Regime

Females (n = 12) were tested in a two-choice scenario for their preference between the scents of the two males of her corresponding pair. Both male scents were presented simultaneously (see below for preference testing protocol). Females were initially tested for preference before males were placed on experimental diets (Week 0); at this point males had been starved for one week. The starvation period allowed for the full passage of field-derived diets through the gut, as confirmed by personal observation of an empty gut as seen by candling. This starvation period reduced any residual effects stemming from differing energy-budgets prior to capture, which is known to vary between males in the wild (Jaeger 1980; Jaeger et al. 1981; Mathis 1991; Gabor and Jaeger 1995). The slight differences in mass between males were calculated for each pair ($\bar{x}$ = 0.05 g ± 0.03 SD), which was used to distribute the scents of the slightly larger males evenly between the left and right side of the chambers for the initial trial. In this way, each side received an equal amount of slightly larger male scents, which minimized any bias for a certain side of the chamber corresponding to size. After testing female preference prior to experimental feeding (Week 0), the male from each pair whose scent the female associated with least was chosen to be the male placed on the high-quality (HQ) diet, while the male whose scent elicited a greater female association was placed on the low-quality (LQ) diet: by doing this females would be required to change their preference from the original male. Therefore, subsequent preference for HQ males was more likely to be a function of diet, instead of the elaboration of an already existing preference.

Each week, feeding of males took place on the day after female preference testing (starting after the initial trial; Week 0). Males on HQ diets were fed 0.1 g of white worms (*Enchytraeus albidus*), while LQ males were fed 12-14 wingless fruit flies (*Drosophila melanogaster*). Based on the mass of adult flies ($\bar{x}$ = 1 mg), this equates to a total mass of ~0.012 g. While the two dietary items have comparable levels of fat and protein (Ivleva 1969; Barker et al. 1998), the "quality" of the diet was based largely upon the proportion of chitinous components. Chitin reduces the digestion efficiency of a prey item (Bobka et al. 1981; Jaeger et al. 1981; Walls et al. 1989; Gabor and Jaeger 1995). Due to the mass of the prey items given, males on HQ diets also obtained a greater total number of calories. After feeding, female preference testing was conducted six days later to allow males time to feed, digest and incorporate the differing nutrition into their physiology. This pattern of feeding and testing was continued until (1) females exhibited a preference for males on HQ diets, and (2) this preference was repeated on the following week.

Female Preference Testing

To assess female preference to male scents, I used a plastic Y-maze with two adjacent chambers at the end of the main arm. The main arm (5 x 11 x 5 cm; W x L x H) had a slit in the lid through which a removable barrier could be inserted. This confined the focal females within the first 5 cm of the main arm for a habituation period prior to testing. The end of the main arm had two adjacent openings (2.5 cm wide) to the left and right chambers (5 x 5 x 5 cm). The floors of the chambers were also displaced vertically (2.5 cm) from the main arm, which served to simulate a natural territory (minus the cover object). The substrate in each chamber was a square piece of paper towel (5 x 5 cm), on which the scents of the source males were placed prior to each trial.

Prior to any of the trials for a given week, male pairs were placed into 5 mL of water ("body wash") in small plastic dishes (5 cm diameter x 1 cm tall) with holes in the lid. All males were given a preliminary rinse prior to the body wash, to eliminate any potential food-derived cues from the skin. The preliminary rinse consisted of holding the males in larval forceps and rinsing all regions of their body for several seconds with deionized water. All males were treated in the same manner during the preliminary rinse. After six hours in the body wash containers, the males were removed and the water from each pair was used as the source of semiochemicals in the corresponding female preference trial. For a given trial, the body wash water from each male of the appropriate pair was placed onto the paper towel in the designated side of the Y-maze. The order of females (and thus male pairs) tested on a given night was randomized each week. The side to receive a given male scent was also reversed each week to control for potential side biases.

To commence the trial, the corresponding female was placed into the end of the main arm of the Y-maze, and the removable barrier was inserted. Females were left to habituate to the chamber for five min after which the removable barrier was lifted and recording commenced. A continuous focal sampling method was employed. "Time-in-proximity" (TIP) was used to indicate female spatial association with each male scent and was defined as the duration of time (sec) spent inside each chamber with the male scent. The number of Nose Taps (NT) was also recorded; nose tapping is a discrete chemoinvestigatory behavior used to obtain non-volatile odors from the substrate (Brown 1968). The behavior consists of distinctly pressing the nasolabial grooves to the substrate, through which the substrate-borne odors are delivered directly to the vomeronasal epithelium (Dawley and Bass 1989). The number of NT is proportional to the degree of investigation of chemical signals, and this behavior is seen frequently in both agonistic and courtship encounters (Jaeger and Gergits 1979; Jaeger 1984; Jaeger 1986). The "latency" to make a choice was recorded as the length of time between the end of the habituation period (lifting the barrier) to when a female entered one of the two chambers. This behavior was used as a measure of how quickly females responded to the male odors. Females were considered fully in (or out of) a chamber when the entire head and both front limbs had crossed the plane of the entrance. Trials lasted for five min, beginning at the end of the latency period (i.e., when a female first entered a chamber). All trials began at 2000 on the day of testing and were carried out under red light to minimize the degree of disturbance.

The behavioral data were not normally distributed (Shapiro-Wilk test and the Kolmogorov-Smirnov test). The data for TIP and NT were analyzed using a two-tailed t-test assuming unequal variance (Welch's t-test) performed with rank transformations to accommodate for a non-normal distribution (Zimmerman and Zumbo 1993; Ruxton 2006). The data for latency were analyzed by a sequential Wilcoxon signed-ranks test. All statistics were conducted in Microsoft Excel. Alpha = 0.05 for all statistics.

Protein Assay of Signaling Glands

Animal Collection and Feeding

Salamanders were collected in May 2009 from Durham, NH to investigate the effect of the experimental diets on the volume of the signal (i.e., protein levels). Animals were maintained in the laboratory under the same conditions as the behavioral study. The salamanders were starved for one week to allow for the complete passage of field-derived diets, at which point they were weighed (g), measured (SVL, TL; mm) and the sex was determined via candling. Prior to experimental feeding, males (*n* = 24) were assigned to twelve size-matched pairs (± 0 mm SVL; 2 mm TL; 0.1 g) to eliminate the effect of initial size. Each male of the pair was placed onto either the HQ or LQ diet. The male to receive each experimental diet was determined by again considering differences in mass between size-matched males of the pair ($\bar{x}$ = 0.06 g ± 0.04 SD): half of the slightly heavier males received the HQ diet, while the other half received the LQ diet. The average mass (prior to feeding) of the LQ males ($\bar{x}$ = 0.87 g ± 0.11 SD) was not significantly different than the HQ males ($\bar{x}$ = 0.84 ± 0.09 SD; t_{22} = -0.33, *P* = 0.74). At this point males were fed their respective diets for six weeks (items and amounts identical to above). After six weeks of experimental feeding, males were re-weighed to determine change in mass as a function of the experimental diets and I surgically removed their mental and postcloacal glands for analysis of protein levels.

An additional group of males (*n* = 22) were also collected at the same time and location for analysis of gland protein concentration. These animals were not experimentally manipulated and glands were assayed for protein levels directly from the field ("field-quality" males). This served as a baseline for realistic levels of variation between males in the field for comparison to experimental groups.

Gland Removal and Protein Assay

For gland removal surgeries, males were anesthetized in 100 mL of 7% diethyl ether for ~10 min (until the loss of the righting-response). Mental and postcloacal glands were surgically excised. The glandular regions of interest were identified based on past morphological and histological analyses (Sever 1976; Simons and Felgenhauer 1992; Simons et al. 1994, 1999; Hecker et al. 2003), as well as personal confirmation via histology. Upon removal, glands were first rinsed in Amphibian Ringer's solution and then placed in 200 µL of 0.8 mM Acetylcholine Chloride in Ringer's solution to help release the granular gland secretions (Hoffman and Dent 1977; Feldhoff 1999). Glands were left in the extraction solution for 60 min, at which point the glands were removed and discarded. After extraction, the solutions were centrifuged (14,000 rpm) at 4°C for 10 min; the supernatant was then transferred and re-centrifuged. This process was repeated for a total of three rounds of centrifugation, after which the proteinaceous solution was frozen at -20°C until protein analysis. The solutions for both glands of all males were tested for protein concentration with a bicinchoninic acid (BCA) protein assay (Smith et al. 1985). All samples were run in a single assay in a 96-well plate to eliminate inter-assay variation. Protein levels for each glandular region were compared between high- and low-quality males. These data were normally distributed and were subjected to a two-tailed Welch's t-test.

Results

Time-course of Female Diet-quality Preference

When males were originally size-matched and had been starved for three weeks (Week 0), gravid females exhibited no significant preference to associate with the scent of either male based on the left or right side of the apparatus (t_{22} = -0.24; P = 0.81). After one week of experimental feeding (Week 1), females spent significantly more TIP to the scents of HQ males (t_{22} = 2.86; P = 0.009; Fig. 1). Females spent over twice the amount of time in association with the HQ scent ($\bar{x}_{HQ}$ = 211.25 sec.; $\bar{x}_{LQ}$ = 77.17 sec.). This same preference was shown after an additional week of experimental feeding (Week 2), when the scents of each male were reversed (t_{22} = 2.31; P = 0.031).

Significant differences in the investigatory behavior of females took slightly longer to manifest (Fig. 2). During the initial test (Week 0), there was no significant differences between the number of NT (t_{22} = 0.11; P = 0.92). There was also no significant difference after one week of differential feeding (t_{22} = 1.60; P = 0.13). However, after two weeks, females exhibited a significantly greater number of NT to the scents of HQ males (t_{22} = 3.52; P = 0.002).

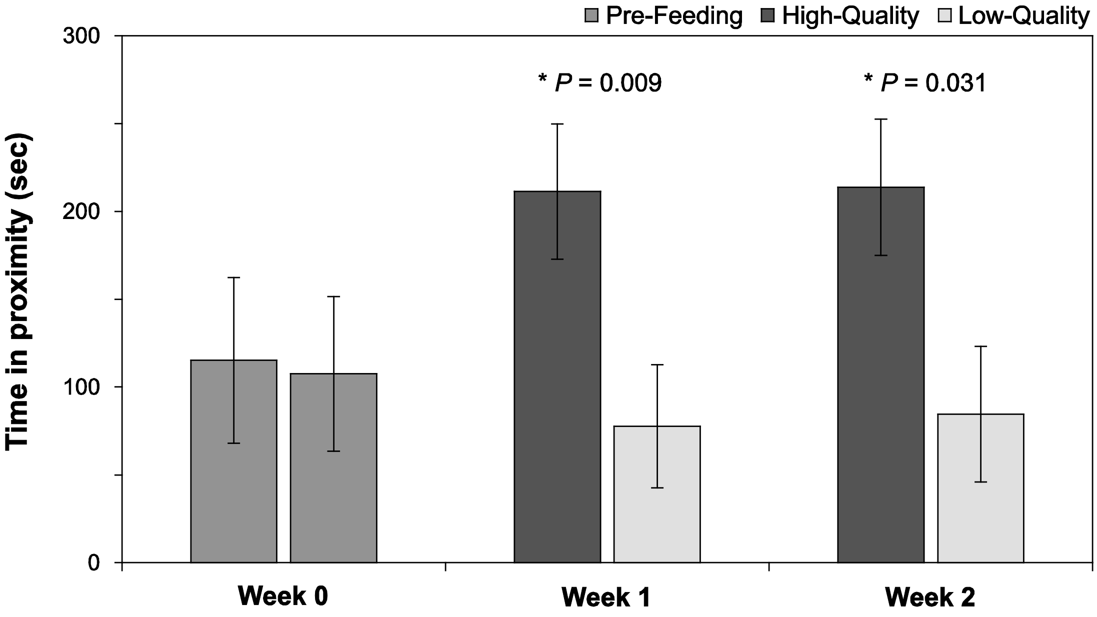

Fig. 1 The time (sec ± SE) that gravid female *Plethodon cinereus* (n = 12) spent in proximity to the scents of males on high-quality (dark) vs. low-quality diets (light), compared to the same size-matched males before feeding (medium)

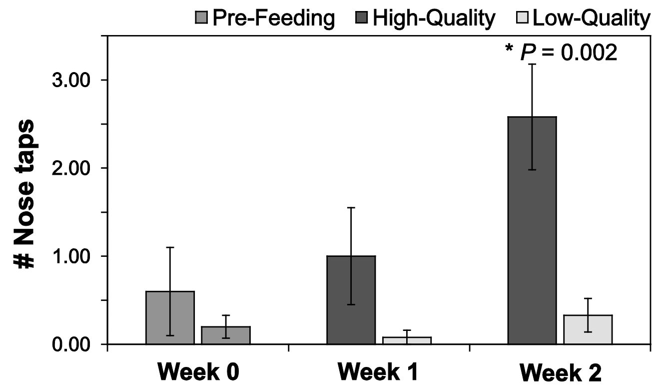

Fig. 2 Investigatory behavior (number of nose taps ± SE) of male chemical signals during the onset of mate preference by female *P. cinereus*

Gravid females responded more quickly to the scents of both males of a pair after experimental feeding had taken place (Fig. 3). After one week of feeding, females responded to the male scents significantly faster (T = 7; $n = 10$; $P < 0.02$). The latency continued to drop after two weeks of feeding, though this decrease was not statistically different than Week 1 ($T = 20$, $n = 11$, $P > 0.05$).

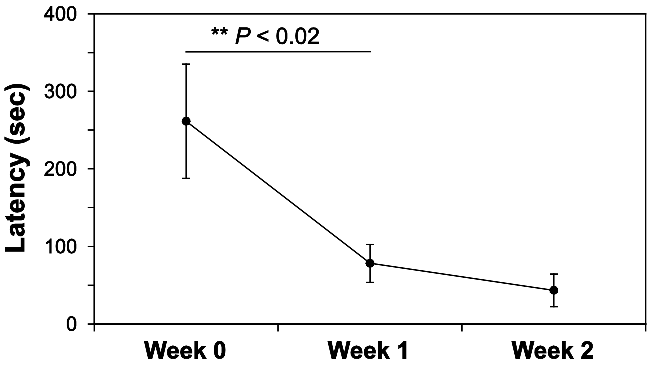

Fig. 3 Latency (sec ± SE) of gravid female *P. cinereus* to make a behavioral preference for males before (Week 0) and after feeding (Week 1 & 2)

BCA Protein Assay of HQ vs. LQ Male Glands

After six weeks of differential feeding, males placed on HQ diets had significantly more protein in the mental gland (MG; $t_{17} = 3.60$; $P = 0.002$) and the postcloacal gland (PCG; $t_{17} = 4.13$; $P = 0.0007$) than males on LQ diets (Fig. 4). The maximum variation in all diet-quality males ranged from 2.9 – 40.6 µg in the MG, and 61.7 – 488.2 µg in the PCG. For both glands, the protein produced by males on HQ diets was double that of males on LQ diets. In all males, the mass of protein in the PCG was an order of magnitude greater. However, the PCG region of interest is also an order of magnitude larger than the MG, so there is not a noticeable difference in the actual density of protein in either gland region.

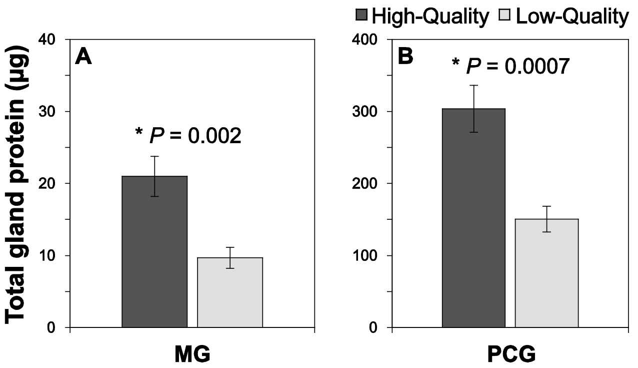

Fig. 4 Total gland protein (µg ± SE) in the mental gland (MG; A) and postcloacal glands (PCG; B) of high- and low-quality male *P. cinereus* after six weeks of differential feeding

The amount of gland protein was directly proportional to the size of the male sender (Fig. 5c-d): larger males were more likely to have more protein in the MG ($r = 0.79$; $P < 0.0001$) and the PCG ($r = 0.79$; $P < 0.0001$). The current energy-budget (mass gain/loss) also had a strong relationship to the amount of gland protein (Fig.5a-b): males on positive energy-budgets were more likely to have more protein in the MG ($r = 0.77$; $P < 0.0001$) and PCG ($r = 0.77$; $P < 0.0001$) than males on negative energy-budgets.

The gland concentration of field-quality males ranged from 9.2 – 26.3 µg in the MG ($\bar{x} = 17.5 \pm 5.02$ SD) and 55.3 – 318.8 µg ($\bar{x} = 152.9 \pm 59.8$ SD) in the PCG. Thus, the maximum variation between males of the sample population is a difference of 17.1 µg in the MG and 263.5 µg in the PCG. While the maximum variation after six weeks of experimental feeding slightly exceeds these levels, females made behavioral choices after only one week. When assuming a linear increase in protein levels over this time, the difference in total protein between HQ and LQ males would be 1.9 µg for the MG and 25.5 µg for the PCG after one week, which falls well within the range of variation observed in field-quality males.

Discussion

Gravid females were able to detect changes in the diet-quality of male pairs through chemical signaling within one week of experimental feeding. Females demonstrated affinities for HQ males by preferentially associating with (Fig. 1) and investigating the HQ scents (Fig. 2) after only a short time in which males of equal initial size could have diverged in their nutritional and energetic condition. These findings are in agreement with several past studies demonstrating that females are capable of differentiating between the scents of individuals on different diets (Beauchamp 1976; Ferkin et al. 1997; Fisher and Rosenthal 2006). However, as noted by Ferkin et al. (1997), relatively few studies have elaborated on the

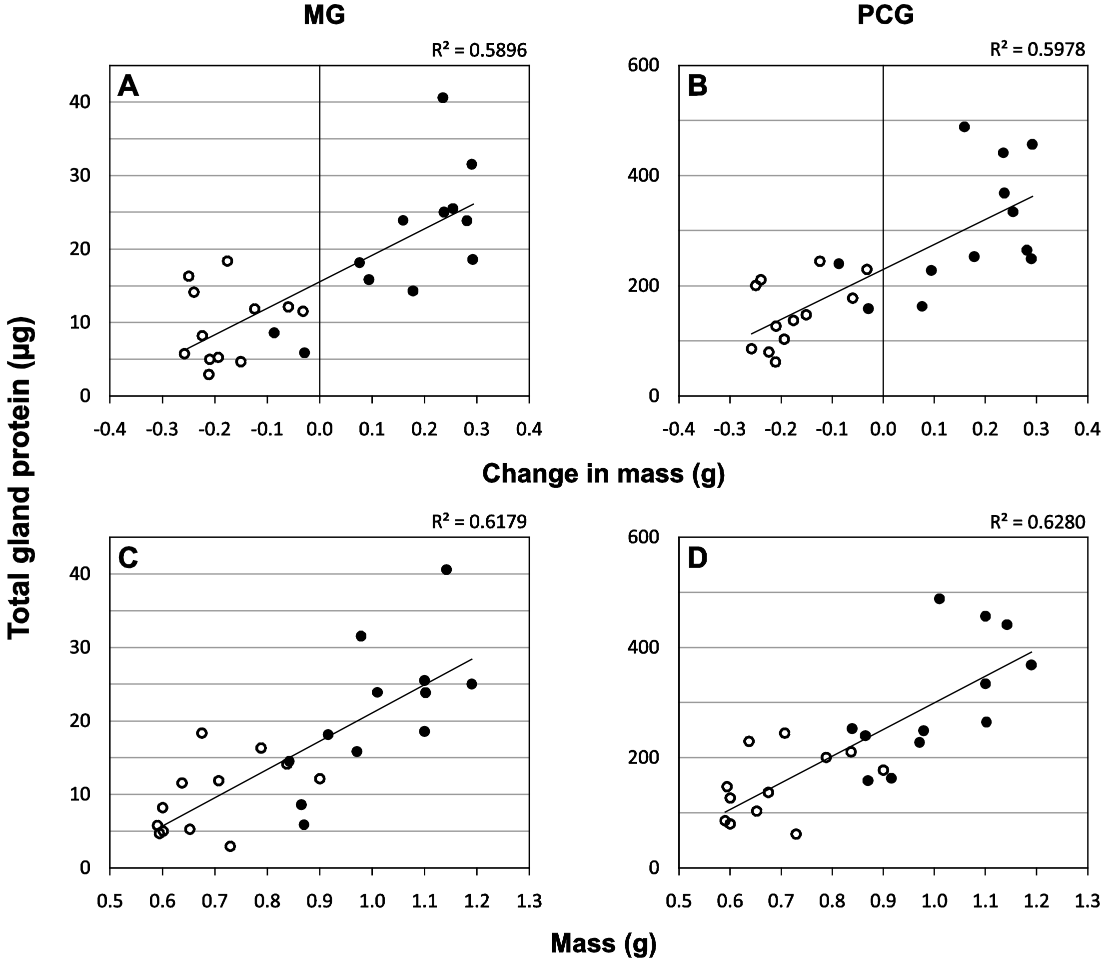

Fig. 5 Total gland protein (μg ± SE) in the mental gland (MG; left column - A and C) and postcloacal gland (PCG; right column - B and D) of male *P. cinereus* as a function of the change in mass (top row - A and B) and the total male mass (bottom row - C and D) after six weeks of differential feeding. Closed circles represent males on high-quality diets, while open circles represent males on low-quality diets

consequences that dietary modulation of chemical signaling can have on mate choice. One such study in red-backed salamanders found that females were able to detect the quality of male diets through olfaction (Walls et al. 1989). However, the source of the odor used for these tests was fecal pellets, so discerning between fecal cues and active signaling is not possible. In addition, Walls et al. (1989) found no substantial female preference to investigate the HQ diets (as measured by NTs). In contrast, I found that (1) fecal pellets were not necessary for detecting diet-quality, but also that (2) female salamanders exhibited both strong association (TIP) and investigatory (NTs) preferences to the scents of males on HQ diets over short feeding periods.

As noted above, past studies have not established whether females are detecting changes in active male signaling *per se*, or simply inferring differences in the diet indirectly through food-derived cues. Admittedly, even in the current experiment it is possible that food-derived cues were being passed into the body wash water (e.g., in urine, or passively diffusing into the water from the skin of the animal). Even so, several aspects of the experimental design allows for a large step in the direction of demonstrating that diet-quality detection may have a true signaling component. For one, the elimination of fecal pellets and substrate-borne odors concurrently eliminates the vast majority of residual food cues (both chemical and visual) that could be utilized by females to detect male diet-quality. Secondly, all males were rinsed prior to placement in the body wash containers; this preliminary rinse would eliminate the majority of residual food particles from the male's home substrate that could have been lingering on the skin. Thus, the observed preference by females was less likely to be due to indirect cues, and appear to be the result of true signaling on behalf of the males.

My results provide evidence that fecal cues are not necessary for inferring information about diet-quality, but they may still be used as supplementary information in this regard. Karuzas et al. (2004) proposed an alternative hypothesis that female "fecal squashing" is a means of gaining information pertinent to foraging, but this hypothesis is not mutually exclusive with a role in mate assessment. Even if female investigation of fecal pellets originally evolved as a means of gathering foraging information, this behavior could have been co-opted as an additional tool for mate assessment. The same behavior would provide information regarding foraging opportunities, in parallel with information about the male's quality that enabled him to obtain access to, defend and capitalize on the high-quality resources.

In addition to eliminating confounding cues (the exclusion of feces and the use of a preliminary rinse), the strongest line of evidence for active chemical signaling of diet-quality is the protein assay data of known signaling glands (Fig. 4 and 5). The original hypothesis that greater energetic resources would result in an increase in secretory protein production was supported, as males on HQ diets possessed greater amounts of protein in both glands tested. These data imply a physiological pathway that translates dietary resources into male-produced chemical signals. Measuring the actual protein content of known signaling glands indicates that dietary resources do in fact translate into a greater volume of secretory products in the glands that are involved in communication pertaining to territoriality and mate assessment.

The amount of protein produced in the mental and postcloacal glands is clearly a function of the overall condition of the sender. Specifically, the amount of secretory protein produced by an individual is a function of their absolute size: larger males make more secretory protein. This is most likely a major mechanism by which chemical signals convey the information of size in plethodontids (Mathis 1990a; Marco et al. 1998). Importantly, the amount of secretory protein produced was also shown to be strongly correlated with the actual current energy-budget of the sender: males that are gaining mass produce more secretory protein than males that are losing mass. As males of each pair were closely size-matched, yet females exhibited strong preferences to associate with HQ males within just one week, the current energy-budget of the sender was most likely what was conveyed to females via the quantity of secretory protein.

One limitation of my results is that the protein assay data were measured from males that had been fed on different diets for a period of six weeks, as opposed to the one week in which females detected differences in male scents. The rationale for this discrepancy in timing stems from logistical constraints of the biochemical analyses of HQ and LQ diets. The males for biochemical analysis were maintained on experimental diets for six weeks in order to detect any differences in the actual composition of the chemical signal. Since salamanders are extremely sensitive to olfactory cues (e.g., odorant concentrations of 10^{-14} M; Kikuyama et al. 1995), it is thought to be possible for females to discern differences between chemical compositions that could not have been detected by use of biochemical techniques. In addition, the need for surgery unfortunately only allowed for measuring gland protein from each male at one time point. Thus, allowing the males a longer period of experimental feeding increased the feasibility of detecting any changes in the biochemical composition of the chemical signal. The variation of protein levels between males after six weeks of feeding reached slightly higher levels than those found in the field, yet females made strong behavioral preferences after only one week. The variation between experimental males after one week of feeding is predicted to fall well within the range of animals in the field. This variation in glandular protein among males from the field demonstrates the importance of possessing high-quality territories in this species. Non-territorial animals ("floaters") maintain lower energy-budgets than territory holders, but even within territorial animals there are discrepancies between male qualities (Jaeger et al. 1981; Mathis 1991; Gabor and Jaeger 1995).

Pheromones from the mental and postcloacal glands are important for a variety of behavioral functions including courtship (MG) and territorial advertisements (PCG). This would suggest that there are behavioral benefits as a result of increased signal production due to quality dietary resources. For example, a larger volume of protein in the PCG will provide stronger scent marks that aid in defense of territorial resources (Simons et al. 1994). Territorial scent marks function as advertisements of male status and are used accordingly in mate assessment by females (Mathis 1990a). In addition, having a larger volume of pheromone in the MG could additionally serve to maximize female receptivity during courtship (Rollmann et al. 1999). However, in order to achieve success in these areas mediated by chemical signals, a male must obtain, maintain and efficiently utilize quality dietary resources.

Females of many plethodontids are known to prefer larger males as mates (Mathis 1991), as larger males are hypothesized to have higher potential fitness. Male salamanders accrue testicular lobes with size/age (Duellman and Trueb 1994), indicating that large males have more energy to invest into reproduction (both pre- and post-courtship). Juveniles will also often reside within the same territory as the parents until reaching maturity, and thus the resource-holding potential of the male could in theory provide prolonged access to resources for the offspring and protection from infanticidal cannibalism (Jaeger et al. 1995; Lang and Jaeger 2000). However, Liebgold and Cabe (2008) found no relationship between relatedness and cohabitation, so this potential benefit is not certain in all cases. However, this same study found that familiarity with adults can positively affect juvenile growth. Thus, in cases where juveniles are residing with parents, having mated with a large male could prove beneficial to offspring growth. Ultimately, being a large male also serves as an indicator of behavioral quality, as large males are more successful in obtaining higher-quality territorial resources and defending them through scent marks and agonistic behaviors (Mathis 1990b, 1991; Gabor 1995). Similarly, male plethodontids are known to preferentially associate with the scents of larger females (Marco et al. 1998) and prefer large females as mates (Verrell 1995), as female size is positively correlated with larger ova and increased clutch sizes (Peacock and Nussbaum 1973; Nagel 1977; Lotter 1978). Thus, the volume of chemical signals produced by females may also be used by males to assess potential mates as well. As size is also important during agonistic encounters (Mathis 1990b), this same mechanism could be used to assess size during intrasexual competition.

While females made strong association and investigatory preferences to HQ males after one week of feeding, they also responded much more quickly to the male odors than the previous week (Fig. 3). Prior to differential feeding (Week 0), both males of a pair were very closely size-matched, on "field-quality" diets, and had been starved for one week. When males were essentially identical in quality and unfed, females took a long time to investigate and did not prefer either male. In contrast, after experimental feeding both males were no longer in a starved condition, but also on different diets. When this was the case, females responded much more quickly and made strong behavioral preferences. Consequently, there were two factors that could have contributed to the decreased response latency of females after experimental feeding: (1) both males being fed instead of starved, and (2) the males differing in their relative diet-quality. In the case of the former, this is certainly in line with the hypothesis that males with more energy invest more into signaling, regardless of their absolute quality (as in Fig. 5a-b). The fact that females were able to respond much more quickly to the male odors on Week 1 indicates that more signal was likely being released, and that these signals were sensed across the entire length of the Y-maze.

Volatile chemicals in plethodontid signaling allow residents to identify intruders present in their territory (Martin et al. 2005), as well as discriminate the sex of the sender and the reproductive status of females (Dantzer and Jaeger 2007a, b). Volatile molecules play essential roles in the pheromone communication of numerous other animal systems (Wyatt 2003), but this study provides the first anecdotal evidence that volatile odors may be involved in the signaling of diet-quality in plethodontid salamanders. Proteins may facilitate this process, given their abundance in these glandular regions and their increased production in response to diet. For example, the major urinary proteins of *Mus musculus* bind small volatile compounds, releasing them over a prolonged period of time; this increases the persistence of the signal in the environment (Hurst et al. 1998).

The glandular region most responsible for conveying diet-quality information is likely to be the postcloacal gland, as this region is responsible for territorial advertisement via scent-marks (Simons and Felgenhauer 1992; Simons et al. 1994, 1997, 1999). Individuals mark the substrate with a characteristic "postcloacal press" behavior, leaving behind secretory products composed largely of proteins and carbohydrates (Hecker et al. 2003; Chouinard 2010). The majority of behavioral experiments examining the types of information conveyed by chemical signals have utilized a medium containing territorial advertisements as the basis for the test (e.g., the animal's home substrate). Thus, the information shown to be conveyed through chemical signals (e.g., sex, species, size, etc.) is likely to have originated in the PCG. Additionally, when individuals first come into contact with one another, the PCG is the primary glandular region contacted by nose taps (Jaeger and Gabor 1993; personal observation). The biochemical composition of these epidermal secretions is also currently being investigated.

In summary, the relationship between an individual and its environment was shown to modulate the chemical signaling system within a very short time period. Changes in the quality of the diet were associated with a change in signal production, which may potentially be facilitated through the MG and/or PCG, but are most likely related to the PCG. The resulting changes in male signaling appear to provide an honest signal for mate quality assessment. The exact role of the chemical substituents produced by these glands in conveying particular aspects of information needs to be tested in future research.

Plethodontid salamanders provide an interesting example of the dynamic interplay between the environment and the quality of an individual, with the added dimension of actually communicating information about this relationship through chemical signals. These data also have broader implications for the fields of sexual selection and evolution, as they imply a more "instantaneous" idea of mate-quality than is often assumed under traditional sexual selection theory. While "good genes" may be an important component of mate quality, the final phenotype will be the result of both genetic and environmental factors that contribute to reproductive success. When considering dietary resources there are multiple components that contribute to fitness, including: (1) the type and abundance of resources available to an individual, (2) the ability of an individual to acquire access to these resources, (3) the efficiency in which one can capitalize on resource availability, (4) the ability of an individual to defend these resources, and (5) the ability of an individual to advertise their status as a function of the above characteristics to attract and persuade mates.

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Ethical Standards

All animal care guidelines and experimental protocols were approved by the Institutional Animal Care and Use Committee (UNH; IACUC # 050802) and all experiments comply with the current laws of the United States of America. All animals were returned to the wild after experimentation, as established in the Animal Care and Use Protocol.

Conflicts of Interest

The author has no conflicts of interest associated with the publication of this research.

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