From:
Avanesyan, A. (2014) Plant DNA detection from grasshopper gut contents: a step-by-step protocol, from tissues preparation to obtaining plant DNA sequences. Applications in Plant Sciences 2(2):1300082

Appendix S3. Protocol for feeding experiments

Developed by A. Avanesyan

All grasshoppers were starved for 24 hours prior to all feeding experiments, which consisted of the following four types.

1) *Feeding experiment with nymph grasshoppers*:

1. Place twelve[1] nymph grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~ 0.3 g total weight) from plants which will be offered to grasshoppers. For example, in this study, *Bouteloua curtipendula* and *Bothriochloa bladhii* plants were used.
3. Put leaves together and wrap the clipped ends of leaves with moist filter paper.
4. Place leaves on the bottom of each container and let grasshoppers feed for 3.5 hours.
5. Randomly choose 7 nymphs which ate the most of leaf tissue and place them separately in new containers. Other grasshoppers should be continued to be maintained in the laboratory for other feeding experiments.
6. Randomly take one grasshopper, put it in a plastic bag, and freeze it immediately at -20^0C.
7. Similarly freeze the rest of the grasshoppers in 2, 4, 6, 8, 10 and 12 hours (h) post ingestion (PI) at -20^0C in separate plastic bags.
8. Freeze samples of leaf tissue (~2.5 cm^2) from both plant species at -20^0C for genetic analysis.

2) *No-choice feeding experiments with adult* M. femurrubrum *grasshoppers*:

1. Place 12 grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~ 0.3 g total weight) from *Bothriochloa bladhii* plants.
3. See steps 3-8 above (in the experiments with nymphs).

3) *Choice feeding experiments with adult* M. femurrubrum *grasshoppers*:

1. Place seven grasshoppers in the same aluminum cage.
2. Prepare a mixture of plants collected on the study plot and place them in a plastic container with water.
3. Place container with plants in the cage with grasshoppers.
4. Let grasshoppers feed on this mixture of plants for two days.
5. Randomly take one grasshopper, put it in a plastic bag, and freeze it immediately at -20^0C.
6. Take other grasshoppers out from the cage and place them separately in small plastic containers.
7. Similarly freeze the rest of the grasshoppers in 2, 4, 6, 8, and 10 h PI at -20^0C in separate plastic bags.

4) *Feeding experiment with adult* M. differentialis *grasshoppers*:

1. Place six grasshoppers individually in small plastic containers.
2. See steps 2-6 in the experiments with nymphs.
3. Freeze the rest of grasshoppers in 1, 3, 8, 10, and 22 hours PI at -20^0C in separate plastic bags. (Two grasshoppers in my study did not eat, so I froze the other four grasshoppers in 0, 1, 3, and 22 hours PI at -20^0C).

[1] Seven nymphs were actually frozen for the DNA extraction; 7+ nymphs (twelve in this study) need to be used in the feeding experiments in case some nymphs do not eat. There can be any number of extra grasshoppers.

Supplies check list:

Plastic containers (7×4.5×5" All Living Things® Critter Totes, PetSmart, Inc., USA)

Aluminum cage (16×16×20" Repti Breeze Aluminum Screen Cage**, Zoo Med Laboratories, Inc., California, USA)**

Small Ziplock plastic bags for freezing