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Effects of internal and external factors on the budgeting between defensive and non-defensive responses in *Aplysia*

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Abstract

Following exposure to aversive stimuli, organisms budget their behaviors by augmenting defensive responses and reducing/suppressing non-defensive behaviors. This budgeting process must be flexible to accommodate modifications in the animal's internal and/or external state that require the normal balance between defensive and non-defensive behaviors to be adjusted. When exposed to aversive stimuli, the mollusk *Aplysia* budgets its behaviors by concurrently enhancing defensive withdrawal reflexes (an elementary form of learning known as sensitization) and suppressing feeding. Sensitization and feeding suppression are consistently co-expressed following different training protocols and share common temporal domains, suggesting that they are interlocked. In this study, we attempted to uncouple the co-expression of sensitization and feeding suppression using: 1) manipulation of the animal's motivational state through prolonged food deprivation and 2) extended training with aversive stimuli that induces sensitization lasting for weeks. Both manipulations uncoupled the co-expression of the above behavioral changes. Prolonged food deprivation prevented the expression of sensitization, but not of feeding suppression. Following the extended training, sensitization and feeding suppression were co-expressed only for a limited time (i.e., 24 h), after which feeding returned to baseline levels as sensitization persisted for up to seven days. These findings indicate that sensitization and feeding suppression are not interlocked and that their co-expression can be uncoupled by internal (prolonged food deprivation) and external (extended aversive training) factors. The different strategies, by which the co-expression of sensitization and feeding suppression was altered, provide an example of how budgeting strategies triggered by an identical aversive experience can vary depending on the state of the organism.

Keywords

Aplysia; Behavioral budgeting; Feeding suppression; Food deprivation; Sensitization

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Conflict of interest

The authors declared no conflict of interest.

1 Introduction

Organisms respond to environmental stimuli by budgeting the expression of their responses to optimize their behavioral output [1]. This principle, originally postulated by Charles Sherrington [2], applies to all animals, including humans. If more energy is required to perform a certain action, such as escape locomotion to avoid a predator, that energy is no longer available for any other actions, such as foraging, growing, or mating [3]. In the most basic form, the behavioral responses made by all organisms can be classified as defensive (i.e., those that increase the survivability of the organism when exposed to danger) and non-defensive (i.e., those that maintain the life of the organism when not exposed to a direct threat) [3,4].

It seems logical for an organism to enhance defensive behaviors at the expense of non-defensive behaviors when threatened, because if they fail to defend themselves they most likely will not survive [5,6]. Indeed, the hierarchical model of behavioral choice, in which defensive responses override other behaviors, including feeding, to prevent predation, was originally shown at the ethological level [7,8] and was later validated at the cellular level [9,10]. There are circumstances, however, in which this choice becomes more complicated, such as when the organism is extremely hungry, or when it is challenged with extreme threat of predation making foraging dangerous for prolonged periods of time. Faced with such situations in which avoiding feeding for prolonged periods could also result in death, an organism must assess the risks associated with foraging to determine if nutritional gain outweighs predation risk [1,5,11,12].

Characterizing how internal and external stimuli differentially modulate the balance between defensive and non-defensive responses over time is, therefore, critical to understand the means by which experience generates and maintains the complex adaptive behavioral output. However, although the effects of aversive experience on defensive and non-defensive responses have been individually studied in vertebrates and invertebrates [3,4,13–15], the relationship between these changes is not yet fully understood. Moreover, previous work on behavioral budgeting primarily describes the interactions between different behaviors and their underlying neural networks in close temporal proximity to stimulus exposure [9,16–20], with little focus on how the budgeting is maintained over time.

In this study, we investigated the process of behavioral budgeting and its maintenance by using a robust learning-induced concurrent modulation of defensive and non-defensive behaviors in the mollusk *Aplysia californica*, which persists from minutes to days depending on the amount of exposure to aversive stimuli [21,22]. In *Aplysia*, exposure to noxious electric stimuli, which mimic the attack of a predator [23], induces a nonassociative enhancement of defensive responses (e.g., withdrawal reflexes and escape locomotion), known as sensitization [24,25], and a concurrent suppression of feeding [21,22,26]. We have consistently observed that feeding is suppressed when sensitization is expressed, but it is unchanged at time points in which sensitization is not expressed [21,22]. The consistent co-expression of sensitization and feeding suppression suggests a strong relationship between these behavioral changes, possibly indicating an underlying mechanistic link.

The goal of this study was to challenge the behavioral budgeting induced by aversive stimuli by attempting to uncouple the co-expression of sensitization and feeding suppression in two different ways. In the first experiment, we examined the effects of a regimen of prolonged food deprivation on the co-expression of sensitization and feeding suppression. Would this regimen shift the balance of behavioral budgeting to favor feeding behavior over defensive withdrawal? With the second experiment, we challenged the co-expression of sensitization and feeding suppression by presenting aversive stimuli using a training protocol that induces sensitization lasting for several weeks (Fig. 3A) [27–29]. Would feeding remain suppressed as long as sensitization is expressed, despite the metabolic strain this would put on the animal? Or, would the behavioral balance shift to allow the organism to feed despite still being in a state of defensive arousal?

2 Materials and methods

General methodologies are described in Sections 2.1–2.4 and experimental designs specific to Experiments 1 and 2 are described in Sections 2.5 and 2.6. In all experiments, the experimenter performing the behavioral tests was unaware of the training history of the animals.

2.1 Animals

Adult *Aplysia californica* (120–190 g) were obtained from South Coast Bio-Marine (San Pedro, CA), and were individually housed in two aquaria (Aquatic Enterprises Inc., WA) of continuously circulating 15 °C aquarium seawater (Instant Ocean) on a 12-h light/dark cycle. Before entering experiments, animals were fed one strip of dried seaweed (3 × 19 cm; 0.5 g; Emerald Cove® Organic Pacific Nori; Great Eastern Sun, Asheville, NC) three times a week [21].

2.2 Measurement of TSWR

At least one week prior to behavioral testing, the posterior portions of the parapodia (i.e., wing-like extensions of the body wall surrounding the mantle cavity) were surgically removed bilaterally (i.e., parapodectomy) to permit full visualization of the siphon withdrawal [29–31]. Prior to the parapodectomy, animals were anaesthetized by placing them under ice for an amount of time (about 20 min for most of the animals tested) sufficient for the animal not to respond to tactile stimuli delivered to the rhinophores [29–31]. This procedure allowed us to remove the parapodia without contractions or the release of ink and/or opaline [21,31]. In the rare cases in which animals secreted ink and/or opaline during or after parapodectomy, they were excluded from the study (7% of animals). The duration of the TSWR was used as a measure of the reflex's strength and was assessed using previously-established testing protocols [21,30–32]. Briefly, the TSWR was measured as the duration of the contraction of the siphon (from the onset of the contraction to the onset of relaxation), elicited in response to a mild stimulus delivered to the tail [30–32]. In all the experiments, a baseline for the TSWR was established by delivering five test stimuli at 10-min intervals (pre-test in Figs. 1A1, Figure 2A, Figure 3A). The five evoked responses were averaged to determine the pre-test TSWR duration [21,30,31]. Animals were excluded from the study if they had a pre-test TSWR duration greater than 10 s (7%), or if they inked and/or secreted

opaline before training (1.5%), as these conditions may be indicative of an animal already sensitized or unhealthy [21,30].

Although the TSWR was measured identically in all the experiments, it was evoked using two different procedures. In Experiment 1, we evoked the TSWR by delivering mild electrical stimuli via two electrodes surgically implanted into the tail [30]. Three days prior to behavioral testing, animals were anaesthetized under ice as describe above, and a pair of Teflon-coated silver wire electrodes (36 Gauge; Medwire Cat# Ag5T, A-M Systems Cat# 786000) was implanted into the tail [21,29–31]. Electrodes were implanted in one randomly-chosen side of the tail, approximately 1 cm from the tip of the tail and 0.5 cm from the midline [29–31]. Animals were excluded from the study if they secreted ink and/or opaline during or after electrode implantation (4%) [21,31]. For each animal, the TSWR was evoked by mild AC electric stimulation of 20-ms duration, using a current intensity set at two times the threshold required to elicit a detectable siphon withdrawal [21,30].

Preliminary results for Experiment 2 revealed that implanted electrodes would not remain in the tail for the duration of the experiment (i.e., 13 days). Therefore, for Experiment 2, we delivered the test stimuli through a hand-held bipolar electrode [33–35]. A pilot study revealed that a brief 2 mA stimulus reliably elicited contractions of the siphon qualitatively comparable to those elicited by implanted electrodes. The variability of responses to 5 test stimuli with a 10-min interstimulus interval was not significantly different than that of responses elicited in pre-test data from a previous data set using implanted electrodes (Mann-Whitney U test: $p = 0.64$; $U = 42.00$). Consequently, in Experiment 2, after an acclimation period, and 30 min before the onset of pre-test measurements, animals were tested with a brief 2-mA electrical stimulus applied with gentle pressure to a predetermined location on one randomly chosen side of the tail. The stimulator was triggered to deliver a 5 s stimulus pulse, during which the wand was touched briefly (300–400 ms) to the tail landmark with gentle pressure. The location of the stimulus was an identifiable landmark of skin coloration (e.g. bright/dark spot) lateral to the midline. If a siphon withdrawal was elicited, stimulus intensity and landmark were used for all pre-test and all post-test measurements for that animal. To allow subsequent stimuli to be applied to the same location, the tail was photographed, and the landmark was annotated using Microsoft One Note. A stimulus intensity of 2 mA was sufficient to elicit the TSWR in all the animals tested.

2.3 Measurement of feeding behavior

The consummatory phase of feeding was assessed by examining the elicitation of bites, defined as openings of the jaws followed by complete cycles of protraction, closure and retraction of the radula [36,37]. Individual animals were placed in a glass bowl containing a seaweed extract solution that is known to reliably elicit biting behavior, and the number of bites was counted during 5-min period [21,38,39]. The seaweed extract was prepared fresh daily by soaking and stirring a sheet of dried seaweed (10.5×19.3 cm) for 30 min in 300 mL of aquarium seawater, and then combining 1 part of the filtrate with 8 parts of aquarium seawater [21,38]. In all experiments, feeding tests were conducted identically prior to (i.e., pre-test) and at different time points after training (i.e., post-tests; Figs. 1B1, Figure 2A,

Figure 3A) [21,39]. A requirement of at least five bites during the pre-test was applied to provide a baseline level of feeding behavior that could be further modified by training [21,22]. Six percent of the animals tested did not meet this criterion and were excluded from the study.

2.4 Sensitization training

Sensitization training was administered using trials (single or multiple), each consisting of 10-s trains of electrical stimuli (500 ms pulses, 1 Hz, 60 mA AC) delivered via a hand-held probe [21,31,32]. The stimuli were applied diffusely along the body wall ipsilateral to the side of tail stimulation [21,31,32]. The number of trials delivered depended on the experimental design, as described in Sections 2.5 and 2.6 below. For all experiments, untrained controls were handled identically to their trained counterparts, but did not receive sensitizing stimuli.

2.5 Experiment 1: Effects of prolonged food deprivation on sensitization and feeding suppression

Animals were randomly assigned to either 2-d or 14-d food deprivation regimens. The 2-d food deprivation regimen is the standard feeding protocol routinely used to analyze the effects of sensitization training on TSWR and feeding. Animals food deprived for 2 days exhibit concurrent sensitization and feeding suppression across different training paradigms [21,22]. The 14-d food deprivation regimen was used to investigate the effects of prolonged food deprivation on the co-expression of sensitization and feeding suppression. Fourteen days is the maximum reported period of food deprivation that *Aplysia* can sustain without signs of health deterioration [36,40]. A pilot study revealed that 14 days of food deprivation induced a small but significant weight loss (change in weight in 14-d food-deprived animals: $-4.09\% \pm 1.73$, $n = 33$; change in weight in 2-d food-deprived animals: $1.63\% \pm 0.97$, $n = 32$; $p < 0.05$; $U = 182.000$; Mann-Whitney U test). This finding aligns with previous work on the abalone *Haliotis kamtschatkana* that can sustain 27 days of food deprivation with minimal loss in body weight [41].

Animals were initially given a piece of seaweed. After the seaweed was completely consumed, the animal was moved into a food-deprivation compartment of the aquarium tank and not fed again until after the experiment was concluded. Regardless of the regimen, behavioral pre-tests began at the end of the food-deprivation period (2-d or 14-d). Although previous work indicates that *Aplysia* can sustain extensive periods of food deprivation [36,40], great caution was taken to monitor the animals' health conditions throughout the experiments. Several factors were used to evaluate the animals' condition. Behaviors that are indicative of distress and health deterioration, such as spontaneous release of ink and/or opaline before treatment (training/no training), flaring of the parapodia with exposure of the gills, or failure to attach to surfaces with the foot were monitored. Health of the animals was monitored throughout the food deprivation period up until the beginning of Experiment 1. No animals in this study were disqualified based on the above monitoring.

Because the effects of prolonged food deprivation on the expression of sensitization and feeding suppression may depend on the amount of sensitization training administered, we

used two protocols: single-trial (Experiment 1A) and four-trial (Experiment 1B) training. The single-trial training is known to induce sensitization and feeding suppression observed 15 min, but not 24 h after training [21,42,43]. The four-trial training is known to induce concurrent long-term sensitization and feeding suppression lasting 24 h, but not 72 h [22,31,44].

2.5.1. Experiment 1A—*Aplysia* went through testing and training according to the protocol illustrated in Fig. 1A1 and B1. Briefly, behaviors were tested prior to, and at the 15 min post-test time point [21], which is representative of the presence of short-term memory [25,42,43]. The elicitation of the TSWR may have short-lasting effects on the subsequent expression of feeding, and vice versa, when these two behaviors are evoked in close temporal proximity [45]. Consequently, it was not technically feasible to measure both TSWR and feeding in the same animal at the 15 min post-test, without the potential bias of interactions between the two behaviors [21]. Therefore, in this experiment, the TSWR and feeding were examined in two cohorts of animals (Fig. 1A1 and B1).

In one cohort of animals, the TSWR was measured during the pretest as described in Section 2.2 (Fig. 1A1). Animals were then randomly assigned to either be trained or serve as untrained controls. Training, consisting of a single trial of sensitizing stimuli, began 30 min after the end of the pre-test. To capture the response 15 min after training, the TSWR was measured using a single 20-ms stimulus [21,46].

In the second cohort of animals, feeding was measured during the pre-test as described in Section 2.3 (Fig. 1B1). Animals were randomly assigned to either be trained or serve as untrained controls. Training began 30 min after the end of the pre-test and consisted of a single trial of sensitizing stimuli. Feeding was measured again 15 min after training [21].

2.5.2. Experiment 1B—In this experiment, the TSWR and feeding were assessed in the same animal. Animals went through testing and training according to the protocol illustrated in Fig. 2A. The feeding pre-test was conducted 30 min after the end of the TSWR pre-test, as this interval is sufficient to rule out any interaction between TSWR and feeding [22]. After baseline TSWR and feeding measurements were taken, animals were randomly assigned to either be trained or serve as untrained controls. Training began 30 min after the end of the feeding pre-test and consisted of four trials of sensitizing stimuli, with a 30 min inter-trial interval. Post-tests for TSWR and feeding were conducted 24 h after training in a manner identical to pre-tests [22]. Measurements of both TSWR and feeding were suspended for those animals (7%) in which one or both electrodes did not remain implanted in the tail between the pre-tests and the post-tests.

2.6. Experiment 2: Effects of extended training on the co-expression of sensitization and feeding suppression

The TSWR and feeding were assessed in the same animal. Animals went through testing and training according to the protocol illustrated in Fig. 3A. Baseline TSWR and feeding measurements were taken, separated by 30 min, and then animals were randomly assigned to either be trained or serve as untrained controls. Training commenced 30 min after the end of the feeding pre-test and consisted of four trials of sensitizing stimuli delivered per day,

repeated at 24 h intervals, for four consecutive days. This protocol is known to induce long-term sensitization lasting several weeks [27–29]. The TSWR and feeding were measured again 24 h, 72 h and 7 days after the end of training (i.e., post-tests).

2.7. Data analysis

For each time point, the change in TSWR duration was calculated as [post-test TSWR duration]/[pre-test TSWR duration] [21,30,31]. Sensitization manifested as a change in TSWR duration > 1 . For each time point, the change in feeding behavior was analyzed as differences in bites, calculated as bites during post-test minus bites during pre-test [21,47,48]. Feeding suppression manifested as a difference in bites < 0 . Data were reported as means $\pm$ SEM and were analyzed using the statistical package of SigmaPlot 11.0 (Jandel Scientific, San Rafael, CA). Statistical significance was set at $p < 0.05$.

When two groups of animals were used, the changes in TSWR duration and differences in bites were compared using the Mann-Whitney U test [21,31,48]. In Experiment 1, which employed four groups of animals, the changes in TSWR duration and differences in bites were compared using the Kruskal-Wallis test (H). This test was followed by the Student-Newman-Keuls pairwise *post-hoc* comparisons (q) to isolate the sources of significance [21,29,46].

In Experiment 2, the co-expression of sensitization and feeding suppression was monitored by measuring TSWR and feeding at multiple post-test time points (24 h, 72 h and 7 days) in trained and untrained animals. In this case, changes in TSWR and differences in bites were compared between trained and untrained groups using repeated measures ANOVA with two factors: training and time [49,50]. This test was followed by the Fisher Least Square Difference pairwise *post-hoc* comparisons to isolate the sources of significance [51,52].

3 Experiment 1: Results and discussion

In Experiment 1, we examined the effects of a regimen of 14 days of food deprivation on the co-expression of sensitization and feeding suppression. The following groups of animals were used: trained/14-day food deprived (T-14), untrained/14-day food deprived (UT-14), trained/2-day food deprived (T-2) and untrained/2-day food deprived (UT-2).

3.1 Results of Experiment 1A

We initially used the single-trial training protocol that induces sensitization and feeding suppression 15 min after training (Fig. 1A1 and B1) [21]. The expression of sensitization was altered by prolonged food deprivation. Analysis of the TSWR revealed an overall statistical significance among the four groups ($H_3 = 9.50$; $p < 0.05$; Fig. 1A2). *Post-hoc* analysis highlights that the T-2 group was significantly different from both the UT-2 ($q = 3.90$, $p < 0.05$) and T-14 groups ($q = 5.06$, $p < 0.05$), and that the T-14 group was not different from the UT-14 group ($q = 2.14$, $p > 0.05$). This result indicates that sensitization was not expressed in animals exposed to the prolonged food-deprivation regimen. The prolonged food-deprivation regimen affected neither baseline TSWR duration nor the current threshold to elicit a detectable TSWR (Table 1). Nor did it alter the sensitivity to sensitizing stimuli

(assessed as the ability to release ink/opaline in response to the noxious electrical stimuli delivered to the body wall during training; Wainwright 2014 personal communication).

In contrast to sensitization, feeding suppression persisted in the animals subjected to prolonged food deprivation. Analysis of feeding revealed an overall statistical significance among the four groups 15 min after training ($H_3 = 16.27$; $p < 0.05$; Fig. 1B2). *Post-hoc* analysis highlights that feeding suppression was observed in both T-14 and T-2 animals (T-14 vs. UT-14; $q = 4.52$, $p < 0.05$; T-2 vs. UT-2; $q = 5.83$, $p < 0.05$). Notably, the prolonged food deprivation regimen did not affect baseline feeding (Table 1). Although feeding was suppressed in T-14 animals, the amount of suppression was greater in T-2 than in T-14 animals (T-14 vs. T-2; $q = 2.81$, $p < 0.05$).

3.2 Results of Experiment 1B

We next investigated the effects of the 14-day food deprivation on the long-term sensitization and feeding suppression induced by the four-trial sensitization training (Fig. 2A) [21,22]. As in Experiment 1A, prolonged food deprivation affected neither baseline TSWR duration, current threshold to elicit a detectable TSWR, nor baseline feeding response (Table 1). Analysis of the TSWR revealed an overall statistical significance among the four groups 24 h after the delivery of the four-trial training ($H_3 = 12.11$; $p < 0.05$; Fig. 2B). Similar to the results obtained with the single-trial training, post-hoc analysis highlights that the T-2 group was significantly different from both the UT-2 ($q = 7.15$, $p < 0.05$) and T-14 groups ($q = 4.44$, $p < 0.05$), and that the T-14 group was not different from the UT-14 group ($q = 1.24$, $p > 0.05$). This result indicates that long-term sensitization was not expressed in animals exposed to the prolonged food deprivation regimen.

As in Experiment 1A, in contrast to sensitization, feeding suppression persisted in the animals subjected to prolonged food deprivation. Analysis of feeding revealed an overall statistical significance among the four groups 24 h after training ($H_3 = 19.09$; $p < 0.05$; Fig. 2C). *Post-hoc* analysis highlights that feeding suppression was observed in both T-14 (T-14 vs. UT-14; $q = 3.93$, $p < 0.05$) and T-2 animals (T-2 vs. UT-2; $q = 5.70$, $p < 0.05$) 24 h after training. Although feeding suppression was expressed in both T2 and T-14 animals, the amount of suppression was greater in T-2 than in T-14 animals (T-14 vs. T-2; $q = 5.95$, $p < 0.05$).

3.3 Discussion of Experiment 1

In Experiment 1, we investigated whether prolonged food deprivation altered the way learned responses to aversive stimuli were budgeted following the delivery of two distinct protocols, single- and four-trial training, which induce concurrent sensitization and feeding suppression lasting minutes and days, respectively [21,22].

Our results indicate that prolonged food deprivation disrupted the co-expression of sensitization and feeding suppression, regardless of the training protocol. In particular, sensitization was not expressed in T-14 animals at any time point (Figs. 1 and 2). The prolonged food deprivation regimen did not alter either the animals' baseline behavioral responses (Table 1) or the sensitivity to sensitizing stimuli, indicating that the lack of sensitization in T-14 animals was not due to a reduced responsiveness to the sensitizing

stimuli. Notably, prolonged food deprivation did not prevent the occurrence of feeding suppression following sensitization training (Figs. 1 and 2).

From a behavioral point of view, our findings reveal an interesting, albeit unexpected, reconfiguration of the balance between defensive and non-defensive responses following prolonged food deprivation. In terms of behavioral budgeting, if the relationship between sensitization and feeding suppression were to change following prolonged food deprivation, one would expect it to change such that the feeding response would prevail, as the need to eat becomes a metabolic priority. This shift in budgeting to favor feeding, even in hostile environments, has been reported numerous times in food-deprived animals. For example, hungry mollusks *Pleurobranchaea* have a low threshold for foraging and may ultimately respond with feeding to noxious stimuli that normally elicit aversive responses [1]. Starving rodents tend to forage in areas that are more susceptible to predators via a hunger-sensitive neural circuit, which suppresses territorial aggression and reduces contextual fear [53]. Effects of food deprivation on the maintenance of a learned change in feeding were also observed in the pond snail *Lymnaea*, in which 5 days of food deprivation abolished the feeding suppression normally observed in response to a conditioned taste aversion task [54].

The shift in feeding caused by food deprivation illustrated in the above examples was largely absent in our results. Although attenuated, feeding suppression was still expressed in food-deprived animals after both single-trial training (Fig. 1) and four-trial training (Fig. 2). Notably, feeding suppression was expressed in food-deprived snails, even in the absence of sensitization (Figs. 1 and 2), a circumstance that seemingly should have taken away the learned “risk factor” and favored feeding over its suppression. These results suggest that the feeding suppression expressed in *Aplysia* at different time points after sensitization training is conserved under a regimen of prolonged food deprivation and appears not to follow the logic of behavioral budgeting. In this regard, the feeding suppression expressed by T-14 animals may represent a form of non-adaptive plasticity [55]. In contrast, the lack of sensitization observed in T-14 animals can be seen as a form of adaptive plasticity, aligned with the logic of behavioral budgeting [3], and consistent with the necessity to prioritize other behaviors over defensive responses, when faced with energy shortage [12,53].

The absence of memory for sensitization aligns with previous evidence that food deprivation can impair learning and memory in vertebrates and invertebrates. For example, in rodents, a feeding restriction regimen, consisting of 50% of the food normally administered, significantly impaired the memory performance in an object recognition test both in the short (1 h) and in the long term (24 h) [56]. In *Drosophila*, starvation regimens of 18–24 h prevented the expression of aversive associative long-term memory (LTM) [57,58]. In particular, following spaced aversive training, starved flies did not form CREB-dependent LTM [57], indicating that *Drosophila* nervous system disables costly memory in order to sustain survival under shortage of food.

Our findings indicate that in *Aplysia* neither short-term memory nor the more “expensive” CREB-dependent long-term memory for sensitization [24,25] are expressed following prolonged food deprivation. A possible explanation for this widespread effect might be that the prolonged food-deprivation regimen disrupts the biochemical machinery responsible for

the induction of sensitization. It is well documented that in *Aplysia* short-term and long-term sensitization are both mediated by serotonin (5-HT), which is released in the neuropil and in the hemolymph upon the exposure to noxious stimuli [25,59–62]. 5-HT synthesis occurs in two steps and involves the action of two enzymes: tryptophan hydroxylase converts the amino acid tryptophan into 5-hydroxytryptophan (5-HTP), which is converted into serotonin by 5-HTP decarboxylase [63]. This process is limited by the availability of tryptophan, which is obtained through the diet [64]. Therefore, tryptophan deficiency may force food-deprived animals to catabolize 5-HT in order to synthesize other molecules and/or to use tryptophan for purposes other than making 5-HT. Over time, prolonged food deprivation may lead to the depletion of serotonin, a condition that is known to render noxious stimuli ineffective in inducing sensitization and its underlying cellular plasticity [59]. In this regard, a reduction in 5-HT synthesis might represent the metabolic substrate underlying the lack of sensitization induced by prolonged food deprivation.

From a biochemical standpoint, the differential effects of prolonged food deprivation on sensitization and feeding suppression may be ascribed, at least in part, to distinct signaling cascades governing these two learned behavioral modifications in *Aplysia*. Recent findings from our laboratory indicate that 5-HT does not mediate either feeding suppression or its cellular correlates [22,65], suggesting that these modifications are brought about by a distinct biochemical pathway. Although further investigation is needed, we may hypothesize that the signaling cascade underlying the training-induced feeding suppression is more resistant to the metabolic consequences of prolonged food deprivation than the 5-HT-mediated pathway responsible for sensitization.

4 Experiment 2: Results and discussion

In Experiment 2, we investigated whether feeding suppression and sensitization remained co-expressed following the delivery of an extended 4-d training protocol [27,28]. Baseline parameters, such as TSWR threshold and duration, as well as feeding response, did not differ significantly between trained and untrained animals (Table 2).

4.1. Results of Experiment 2

We first analyzed the expression of sensitization at three time points after the end of the extended training: 24 h, 72 h and 7 days. A repeated measures ANOVA on the change in TSWR duration in the trained and untrained groups revealed a significant effect of training ($F_{(1,2)} = 27.19$; $p < 0.05$). *Post-hoc* analysis highlighted that sensitization occurred at all the three post-test time points (Fig. 3B): 24 h ($p < 0.05$), 72 h ($p < 0.05$), and 7 days ($p < 0.05$). We next examined the expression of feeding suppression at the above time points after the end of the extended training. A repeated measures ANOVA on the difference in bites in the trained and untrained groups revealed a significant effect of training ($F_{(1,2)} = 5.946$; $p < 0.05$). *Post-hoc* analysis highlighted that feeding suppression occurred only at the 24-h time point ($p < 0.05$), but no longer at either the 72-h ($p = 0.26$) or the 7-day time points ($p = 0.95$; Fig. 3C). These findings indicate that sensitization and feeding suppression were co-expressed 24 h after training. However, a dissociation between these two behavioral changes

was observed beyond the 24-h time point, with feeding returned to baseline at 72 h while sensitization was observed for up to 7 seven days.

4.2. Discussion of Experiment 2

With Experiment 2, we challenged the co-expression of sensitization and feeding suppression by using a training protocol that is known to induce sensitization lasting for several weeks (Fig. 3A) [27–29]. Would feeding remain suppressed as long as sensitization is expressed, or would it resume earlier? From an adaptive standpoint, augmenting defensive responses and suppressing a non-defensive behavior, such as feeding, reflects the animal's requirement to budget its behavioral output following exposure to aversive stimuli [1,3]. However, this strategy carries with it the cost of reducing energy supply due to diminished food intake and, therefore, cannot be sustained without the risk of becoming maladaptive. Our results support this idea and indicate that, under circumstances of heightened exposure to aversive stimuli, sensitization and feeding suppression are co-expressed only at the 24-h time point after training, with a reconfigured behavioral budget at 72 h in which feeding is resumed while the animal remains sensitized for up to seven days (Fig. 3).

Combined with previous results [21,22], our findings highlight different behavioral budgeting strategies depending on the amount of aversive training. With the four-trial training, sensitization and feeding suppression follow the same time course [21,22]. Conversely, with the extended training, sensitization and feeding follow different time courses: feeding resumes at 72 h (Fig. 3C), while the animal is still sensitized (Fig. 3B).

In *Aplysia*, the four-trial training and the extended training produce long-term forms of sensitization that are temporally and mechanistically distinct. The four-trial protocol induces sensitization observed at 24 h, but not at 72 h after training [22,44], whereas the extended protocol induces sensitization lasting for several weeks [27,28]. The sensitization induced by four-trial training is sustained by long-term increase in the excitability of sensory neurons [31,32] and long-term synaptic facilitation of pre-existing sensory-motor synapses [31]. The sensitization induced by the extended training is sustained by long-term synaptic facilitation, which is caused, at least in part, by persisting structural modifications that include formation of new synaptic contacts between sensory and motor neurons [27–29,66].

In contrast to long-term sensitization, the duration of the co-expressed feeding suppression does not appear to be influenced by the amount of aversive training. Four-trial training [22] and extended training (Fig. 3) induced feeding suppression with analogous time courses. Although future investigations are required, these results suggest that the molecular machinery underlying the maintenance of the long-lasting sensitization induced by extended training may be lacking in the feeding neural circuit. This scenario would be consistent with the nature of feeding behavior, which, for survival, cannot remain suppressed for a prolonged time.

5 Conclusions

In this study, we investigated whether the budgeting between defensive and non-defensive behaviors induced by aversive stimuli and its maintenance are influenced by internal and

external factors in *Aplysia*. Our findings indicate that this behavioral budgeting was reconfigured and stored differently following either prolonged food deprivation or extended sensitization training. Interestingly, neither manipulation prevented the occurrence of the training-induced feeding suppression normally observed, and, in the case of the extended training, did not alter its time course. The different strategies, by which the co-expression of sensitization and feeding suppression was altered, provide an example of how budgeting strategies triggered by an identical aversive experience can vary depending on the state of the organism.

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A1. TSWR **B1. Feeding** **A2. 15-min post-test** **B2. 15-min post-test** **Fig. 1.**

In Experiment 1A, prolonged food deprivation prevented the expression of sensitization but not the expression of feeding suppression following single-trial training. The TSWR (A1) and feeding (B1) were measured prior to, and 15 min after training. Sensitization was absent in T-14 animals but was observed in T-2 animals (A2). Conversely, feeding suppression was expressed in both T-14 and T-2 animals (B2). In this and in the following figures, values are expressed as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$.

Fig. 2.

In Experiment 1B, prolonged food deprivation prevented the expression of sensitization but not the expression of feeding suppression following exposure to the four-trial training protocol. TSWR and feeding were tested before and 24h after training (A). Twenty-four hours after training, sensitization was absent in T-14 animals, but was observed in T-2 animals (B). Conversely, feeding suppression was expressed in both T-14 and T-2 animals (C).

Fig. 3.

In Experiment 2, the co-expression of sensitization and feeding suppression was disrupted by an extended training protocol. TSWR and feeding suppression were measured prior to, and 24 h, 72 h and 7 days after extended training (4 trials per day $\times$ 4 consecutive days; A). Sensitization (B) and feeding suppression (C) were only co-expressed 24 h after training. Feeding suppression was no longer observed 72 h or 7 days after training (C), whereas sensitization was still expressed at these two time points (B).

Analysis of the pre-test baseline parameters in Experiment 1. Values are expressed as mean $\pm$ SEM. Comparisons were made between animals that were food deprived for either 2 days (2-d FD) or 14 days (14-d FD). The Mann-Whitney U test was used to compare changes between groups. Statistical significance was set at $p < 0.05$.

Table 1

Experiment	Parameter	Animals (n)	Feeding regimen		P	U
			2-d FD	14-d FD		
1A	TSWR threshold (mA)	2-d FD = 22; 14-d FD = 22	1.01 $\pm$ 0.16	1.18 $\pm$ 0.142	0.36	202.50
	TSWR duration (s)	2-d FD = 22; 14-d FD = 22	4.38 $\pm$ 0.45	3.67 $\pm$ 0.53	0.16	181.00
	Number of bites	2-d FD = 42; 14-d FD = 42	22.12 $\pm$ 1.40	19.10 $\pm$ 1.31	0.10	698.50
1B	TSWR threshold (mA)	2-d FD = 28; 14-d FD = 28	0.958 $\pm$ 0.11	1.12 $\pm$ 0.11	0.13	299.50
	TSWR duration (s)	2-d FD = 28; 14-d FD = 28	3.47 $\pm$ 0.21	3.73 $\pm$ 0.26	0.68	366.00
	Number of bites	2-d FD = 28; 14-d FD = 28	20.00 $\pm$ 1.62	16.86 $\pm$ 1.59	0.20	312.50

Table 2

Analysis of the baseline parameters in Experiment 2. Values are expressed as mean $\pm$ SEM. In this table, “trained” and “untrained” refer to the groups to which the animals were assigned after the pre-test baseline parameters were taken. The Mann-Whitney U test was used to compare changes between groups. Statistical significance was set at $p < 0.05$.

Parameter	Animals (n)	Trained	Untrained	P	U
TSWR duration (s)	Trained = 10; untrained = 10	3.13 $\pm$ 0.242	3.59 $\pm$ 0.33	0.33	22.00
Number of bites	Trained = 10; untrained = 10	13.88 $\pm$ 3.19	21.25 $\pm$ 2.58	0.13	17.50