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Effects of age on feeding behavior and chemosensory processing in the pond snail, *Lymnaea stagnalis*

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Abstract

This study used behavioral and electrophysiological techniques to examine age-related changes in the feeding behavior and chemosensory processing in the pond snail, *Lymnaea stagnalis*. Increasing age was associated with a 50% decrease in long-term food consumption. Analysis of short-term sucrose-evoked feeding bouts showed an age-related increase in the number of animals that failed to respond to the stimulus. Of the animals that did respond increasing age was associated with a decrease in the number of sucrose-evoked bites and a increase in the duration of the swallow phase. These changes were observed with both 0.01 and 0.05 M sucrose stimuli but were not seen when 0.1 M sucrose was used as the stimulus. Electrophysiological analysis of the chemosensory pathway in semi-intact lip-CNS preparations failed to demonstrate a significant change in the neuronal information entering the cerebral ganglia from the lips via the median lip nerve, but did demonstrate an age-related deficit in the neuronal output from the cerebral ganglia. This deficit was also dependent on the sucrose concentration and mirrored the concentration-dependent changes in feeding behavior. In summary, aging appeared to affect central but not peripheral processing of chemosensory information and suggests that this deficit contributes to the age-related changes in feeding behavior.

Keywords: Neuronal aging; Central pattern generator; *Lymnaea*; Feeding; Chemoreception

1. Introduction

Rhythm generating circuits are present in a wide variety of organisms as diverse as the mollusks and humans [4,10,16,38]. They are responsible for generating the motor patterns that allow organisms to carry out some of their most basic functions, such as breathing, feeding and locomotion. Over 30 years of research has helped us understand the properties of these circuits and how they can be modulated to generate a flexible motor output to suit the ever changing needs of the organism. The ability of the neural networks to generate a basic rhythm is due to a combination of the endogenous properties of the neurons that comprise the circuit and their connectivity with each other. The basic motor pattern can be altered by modulatory neurons that influence the endogenous properties of the pattern generating neurons, the strength of the connections between neurons in the network and their general level of excitability [for reviews see 1,4,26,27].

Much of our knowledge about the functioning of pattern generating circuits has arisen from work on the CNSs of relatively simple organisms, such as mollusks [e.g. 4] and crustacea [28,40]. More recent work on vertebrates has examined the networks of neurons that control locomotion in the *Xenopus* tadpole [37], lamprey [17] and most recently in the neonatal rat and mouse spinal cord [32]. Results from these studies has corroborated the invertebrate work demonstrating that the basic principles of how rhythm generating circuits are

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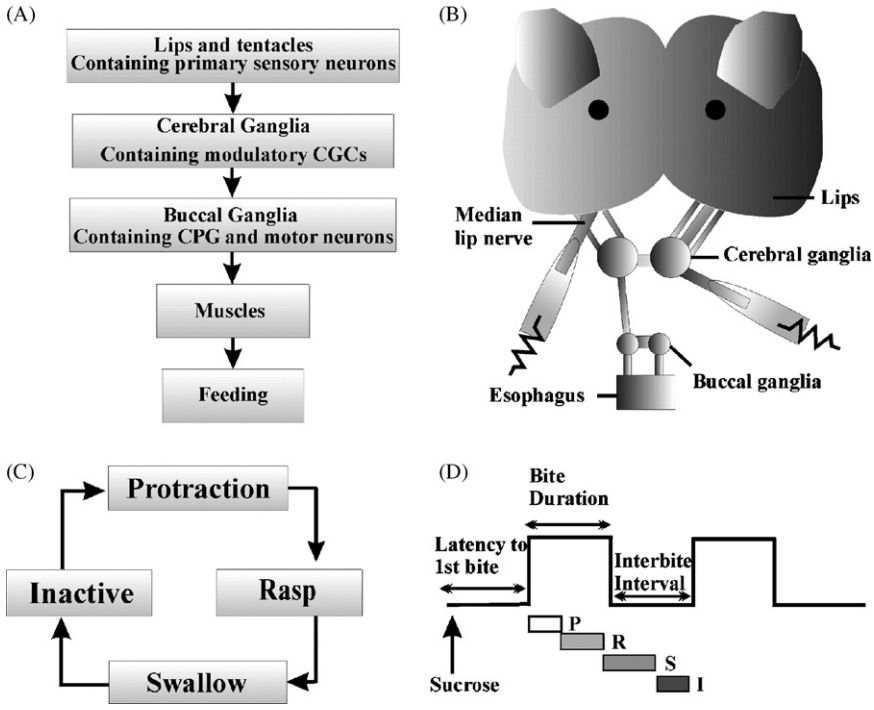

Fig. 1. Diagrammatic representation of the snail feeding system. (A) Circuit diagram illustrating key components regulating the feeding behavior of the pond snail, *Lymnaea*. Food stimuli are detected by sensory neurons located in the lips. Information is then transmitted to the modulatory cerebral giant cells (CGCs) in the cerebral ganglia and the central pattern generating circuit (CPG) in the buccal ganglia. Rhythmical activity generated in the CPG then excites the motor neurons that in turn drive the activity of muscles to generate a feeding rhythm. (B) Diagrammatic representation of the semi-intact lip-CNS preparation, illustrating the lips connected to the cerebral ganglia. Extracellular recordings were made using suction electrodes from a median lip nerve and the contralateral cerebrobuccal connective. (C) Each feeding cycle consists of a series of active phases. During protraction the mouth is opened and the radula protrudes from the mouth. During the rasp phase, the radula is rotated forwards scraping at the food substrate. Finally during the swallow phase, the radula is rotated backwards forcing food into the esophagus. There is also an inactive phase between the end of swallow and the start of the next protraction phase. (D) Diagrammatic representation of a typical computer-derived feeding trace illustrating the main feeding parameters measured. Horizontal bars illustrate the relationship between these parameters and the three active phases (protraction (P); rasp (R); swallow (S)) and one inactive phase (I) of each feeding cycle.

constructed and modulated has remained unchanged throughout evolution.

To date no work has directly examined how the aging process affects the electrophysiological and biochemical properties of these rhythm-generating circuits and how these changes may affect their behavioral output.

We have chosen to examine the effects of aging on the rhythm generating circuit that controls the feeding behavior of the mollusk, *Lymnaea stagnalis*. This model system was chosen as it has previously been used successfully as a model for studying the basic biology of aging [for review see 48], but more importantly, the rhythm generating circuit regulating feeding has been well described. Over 30 years of research has shown it to consist of sensory, motor, central pattern generating (CPG), and modulatory neurons, whose synaptic connectivity is known in great detail [4,14] (Fig. 1). Our ability to interpret the data presented in this paper relied on our previous knowledge of the *Lymnaea* feeding network and its control of the feeding behavior [reviewed in 4]. Food is initially sensed by the animal using primary sensory neurons located in the lips [45,52]. Information about the food stimulus is sent to the cerebral ganglia via the three nerves the median lip nerve (mln), superior lip nerve (sln) and tentacular nerve (tn). On entering the cerebral ganglia, the signal is believed to be processed before passing down the cerebrobuccal connective to the buccal ganglia where it activates the feeding circuitry [45] (Fig. 1B). Food ingestion in *Lymnaea* involves the rhythmic scraping of food by the toothed radula, which is enclosed within a complex muscular structure called the buccal mass [3]. Rose and Benjamin [38] recognized that each feeding cycle consists of three active phases, namely protraction, rasp and swallow and an inactive phase in which buccal muscle movement was silent (Fig. 1C). Motor neurons within the buccal ganglia of the CNS fire in one of the three phases of the feeding cycle and examples of protraction, rasp and swallow motor neurons are known [5,6,38]. The main rhythm generating neurons are three populations of buccal interneurons called the N1, N2 and N3 cells. They fire in sequence (N1 → N2 → N3), and through their connections with the buccal motor neurons drive the three phases of the feeding cycle (e.g. N1, protraction; N2, rasp; N3, swallow). The motor neurons in turn can contribute to the maintenance of the rhythm via electrotonic connections predominantly with cells active in the same phase of the feeding cycle [43]. It is now realized that there are subtypes of each of the three N cell types,

all of which are essential to generate a full feeding rhythm [7,11,47].

In addition to the main rhythm generating neurons, the CNS of *Lymnaea* also contains modulatory neurons whose role is to shape the rhythm to meet the changing needs of the animal. In the buccal ganglia, a single neuron, the slow oscillator (SO) is capable of regulating the frequency of the feeding rhythm [12,50], while in the cerebral ganglia the paired cerebral giant cells (CGCs) allow the animal to respond to a food stimulus and can also control the frequency of feeding movements [49,51]. In addition to understanding the connectivity and function of many of the neurons involved in controlling the feeding behavior of the animals, many of the neurotransmitters used by the individual cell types are also known. Thus, the SO utilises acetylcholine as its main transmitter as do the N1 cells [13,50]. The N2 cells utilise glutamate as their main transmitter [7], while the CGCs use 5-hydroxytryptamine [5-HT; 29]. Based on previous work in a related mollusk the majority of the motor neurons are likely to be cholinergic [9].

The experiments described in this paper provide an initial characterization of the effects of increasing age on the feeding behavior of the pond snail, *L. stagnalis* and the putative role that changes in the processing of the chemosensory signal play in regulating these changes.

2. Methods

2.1. Experimental animals

All animals were bred in house at the University of Brighton. Animals were kept in large tanks at 18–20 °C on a 12 h light/dark cycle in copper-free tap water. They were fed on alternate days with either lettuce or fish flakes (Tetra UK Ltd.). Animals were kept in groups of up to 600 in large circulating tanks at a stocking density of approximately one snail per litre.

2.2. Determination of survival curves

Large tanks were seeded with up to 25 egg masses, which hatched within 2 weeks of seeding. Upon reaching 3 months of age, the numbers of animals in the tanks were reduced to 600 and deaths recorded at two weekly intervals. Records continued for periods of up to 15 months.

2.3. Measurements of changes in long-term food consumption

In order to determine the effects of age on long-term feeding, animals of different ages were isolated in individual holding tanks maintained within the larger breeding tank at 18–20 °C on a 12 h light/dark cycle. Individual tanks had gratings in the bottom to prevent coprophagy. Animals used in this study were 3–4 months old (young), 6–7 months old (middle aged) and 11–12 months old (old). Three to four months was chosen for the young animals as it represented the earliest age when the animals were sexually mature. Six to seven months was chosen for the middle aged at it represented the time at which 40–50% of the population had died. Eleven to twelve months was chosen to represent old age as this was the period when typically 85% of animals had died. Utilization of these age groups ensured that there were sufficient animals of each age to perform meaningful experiments. Twenty-five animals in each group were isolated and given continuous access to lettuce for a 5 week period. Following this period the total amount of food consumed by each animal was calculated and the corresponding mean for each age group determined.

2.4. Measurement of changes in short-term feeding behavior

The effects of age on short-term feeding were examined using a method previously described by Staras et al. [44]. Briefly, animals were removed from their home tank and maintained in smaller tanks in the experimental laboratory in copper-free tap water for 7 days with free access to lettuce. Laboratory temperatures were between 22 and 23 °C and animals were maintained on a 12 h light/dark cycle. Animals were starved overnight prior to experimentation. Animals from the three different age groups were tested by placing them in a petri dish filled with 90 ml of copper-free tap water. The time taken for them to emerge from their shells (both tentacles visible) was recorded after which 5 ml of tap water was pipetted around the lips of the animal and feeding movements recorded over the next 2 min. At the end of the 2 min period, 5 ml of sucrose (Sigma–Aldrich, UK; final bath concentration 0.01 M, dissolved in copper-free tap water) was added to the dish and feeding movements recorded for a further 2 min. An amount of 0.01 M sucrose was chosen as previous work by Kemenes et al. [22] demonstrated that this concentration was capable of evoking feeding responses in 100% of young animals and unlike lettuce was unaffected by seasonal variations in quality and taste. Feeding parameters were recorded using a software package produced by Staras [44]. These were the latency to first bite, bite duration, inter-bite interval and the frequency of sucrose-evoked biting movements (Fig. 1D).

Animals were exposed to an initial water stimulus to control for contributions the vehicle may have made to feeding and it was our aim to subtract the bites in water from those evoked by sucrose to get a true value for the ability of sucrose to evoke feeding. However, analysis of the bites recorded in water showed them not to be correlated to the number of sucrose-evoked bites, suggesting that the bites in water and those evoked by sucrose were independent events. In a series of additional control experiments animals from all three age groups were exposed to an initial water stimulus followed 2 min later by either a second water stimulus or application of 0.01 M sucrose. A two-way ANOVA of the bites recorded during each treatment showed clear effects of both age and stimulus type but more importantly demonstrated

a strong interaction between the effects of age and stimulus type on the number of recorded bites. These data confirmed that bites recorded in water and those in sucrose were independent events and therefore we have presented the actual sucrose-evoked bites in this paper rather than subtracting the vehicle controls. Additionally, control experiments were performed to determine whether age-related changes in the level of satiety were contributing to alterations in feeding behavior. To test this animal's were fed ad libitum until the commencement of the experiment and tested using the short-term feeding protocol. A second set of controls were performed to examine possible changes in the feeding behavior that may have occurred as a consequence of age-related changes in the body mass of the animals. To test for this possibility, the short-term feeding behavior of groups of 6–7 month old animals that either weighed between 2 and 3 or 4–5 g (equivalent to the range of sizes more normally seen in the 3–4 and 11–12 month groups, respectively) was examined as detailed above.

2.5. Video analysis of short-term feeding behavior

Short-term feeding experiments provided us with information concerning changes in the durations of the bite and the inter-bite interval. However, the bite duration is made up of a combination of both protraction and rasp while the inter-bite interval consists of the swallow and inactive phases (Fig. 1D). Therefore to provide information about how the durations of each of the individual phases of the feeding cycle were altered with increasing age, frame by frame video analysis of sucrose-evoked feeding rhythms was carried out. This was important because the neurons responsible for controlling each of these phases have been well described and therefore a detailed analysis of the durations of the individual phases of each feeding experiment would provide information about the neurophysiological correlates of aging. The protraction phase was defined as the time period from the initiation of mouth opening until the start of the forward rotation of the radula. Rasp was defined as the period between the first forward rotation of the radula and the start of the backward rotation of the radula. Swallow was defined as the period between the first backward rotation and the cessation of muscular activity. The inactive phase was defined as the period when no muscular activity could be observed (see Fig. 1C).

2.6. Effects of varying sucrose concentration on short-term feeding responses

We were keen to determine whether varying the sucrose concentration altered the properties of the evoked feeding behavior and whether these changes were age-dependent. To test for this standard short-term feeding experiments (see Section 2.4) were performed on young and old groups of animals. Briefly, animals were initially exposed to an application of 5 ml of water followed 2 min later by 5 ml of a sucrose solution (final bath concentration of 0.01, 0.05 or 0.1 M). The % non-responding animals were recorded along with the standard feeding parameters described in Section 2.4.

2.7. Extracellular recording from the median lip nerve and cerebro-buccal connective in semi-intact lip nerve preparations

Electrophysiological experiments were performed to examine age-related changes in the electrical responses of chemosensory pathway to sucrose applied to the lips of both young and old animals. The methods have previously been described by Straub et al. [45]. Briefly, these experiments consisted of using a semi-intact preparation in which the lips were attached to the CNS by means of the three lips nerves. The head of the snail plus the central nervous system was carefully dissected from the rest of the animal making sure that the superior and median lip nerves and tentacle nerves were left intact (Fig. 1B). All remaining peripheral nerves were cut. The semi-intact preparation was pinned in a Sylgard-lined recording chamber and a local superfusion pipette was placed in front of the mouth region for constant superfusion of the lips with fresh *N*-[2-hydroxyethyl]piperazine-*N*′-[ethanesulphonic acid] (HEPES)-buffered saline, composition in mM NaCl 50; KCl 1.7; $CaCl_2$ 4; $MgCl_2$ 2; HEPES 5; pH 7.8 at a rate of approximately 1 ml min^{-1}. Excess saline was removed from the opposite end of the recording chamber via a suction line. A set of electromagnetic valves that were controlled by the recording software enabled rapid switching of the superfusion system between the saline stream and sucrose solutions (0.01, 0.05 and 0.1 M). All sucrose solutions were made up in HEPES-buffered saline, and perfused randomly over the lips for 30 s with inter-stimulus intervals of 10 min between successive applications. Perfusion of dye across the lips and a series of preliminary experiments where 0.1 M sucrose was applied directly to the CNS excluded the possibility that sucrose was directly affecting the firing properties of key neurons in the CNS.

Two independent extracellular suction electrodes were used to record activity from cut nerves. One electrode was used to record the activity of a median lip nerve in order to detect information passing to the cerebral ganglia from the primary sensory neurons located in the lip musculature. Both light microscope [45] and ultra-structural studies [52] have shown that the primary sensory neurons in the tentacles and the lips appear to belong to a common population of cells. Further in a set of preliminary experiments, application of sucrose to the lips evoked similar response patterns in all three nerves. These data infer that the information carried by each nerve is similar and therefore we limited our detailed analysis to recordings from the median lip nerve. The second was used to record activity in the cerebrobuccal connective (cbc; Fig. 1B). In order for chemosensory information to activate the feeding circuitry located in the buccal ganglia information must pass from the cerebral ganglia down the cbc. For simultaneous recording of one cbc and one mln, a cbc was cut close to the buccal ganglia and the contralateral mln was

cut close to the cerebral ganglia. Each of the two nerves were then sucked into a separate glass suction electrodes. Signals from the extracellular suction electrodes were differentially amplified using Neurolog 104 pre-amplifiers (Digitimer; gain 10000×, ac coupled, time constant 0.1 s). The amplified signals were low pass (500 Hz) and notch (50 Hz) filtered using Neurolog 125 filters (Digitimer) before they were digitized at a sampling rate of 2 KHz using an Axon TL-1 (Axon Instruments) interface and stored on a PC for subsequent analysis. Offline analysis of the extracellular records was performed using Spike 2 software (CED). Individual spikes in the extracellular records were detected after rectification of the filtered data. The threshold for spike detection was set manually so that it was approximately 20% larger than the peak noise amplitude and peaks that were separated by less than 10 ms were considered as one event to avoid multiple counting of biphasic events. The total number of events for each record was counted in 2 s bins for 210 s starting 60 s prior to a stimulus application. The count in each bin was baseline corrected by subtracting the average number of events for the 15 bins prior to stimulus application from each bin. The baseline subtracted data for equivalent experiments was averaged and plotted giving the time course of the net average change in nerve activity in 2 s bins for each stimulus. In all preparations, a level of background activity that had both tonic and phasic components was recorded in both the mln and cbc.

2.8. Data analysis and presentation

Survival curves were plotted by determining the number of animals that died per 2 week period and expressing these as a % of the number of animals surviving in the previous 2 week period (account was taken of any animals that were removed for experimentation during any 2 week period). This value was then subtracted from the previous % survival figure giving the % survival for that time point. Survival curves were then fitted with a Weibull function (Origin, v6.0).

Short-term feeding data was analyzed as follows. Animals that failed to emerge from their shells within 120 s of being placed in the test arena were deemed to be "non-emergers". Those that emerged but did not respond to sucrose application within 120 s were deemed to be "non-responders". Age differences for these two groups of animals were determined using a χ^2-analysis (Minitab). The remaining animals that did respond to the sucrose stimulus were analyzed as follows. For emergence time, latency to first bite and number of bites, values were taken for each animal within a particular age group, the mean calculated and compared using either an unpaired *t*-test (Excel; two groups of data) or a one-way analysis of variance followed by a post-hoc Tukey test (Minitab) if more than two groups were analyzed. Values for the inter-bite interval and bite duration were analyzed by calculating the mean times of all the bites from an individual animal and then averaging the values from all the animals within the group to obtain a group time that was statistically analyzed using either a *t*-test or ANOVA (see above).

Comparisons of the electrical activity in the mln and cbc, were achieved by summing the net change for each 2 s bin during the 30 s of stimulus application. This gave a total net change for each animal. These values were then compared using *t*-tests.

3. Results

3.1. Determination of the survival characteristics of the snail colony

Fig. 2 shows a plot of the survival curves for four different batches of snails kept in different tanks, with tanks seeded with egg masses at different times throughout the year. The data from each of the four batches could be fitted with a Weibull function and the mean 50% survival time for the four batches was determined as 215.2 ± 10.9 days. Comparison of the survival curves from the four batches using an ANOVA showed that there were no significant differences between the groups ($p > 0.05$), demonstrating consistency in the breeding and maintenance program. The maximum survival time for animals housed under these conditions was 15 months.

3.2. Long-term food consumption

Increasing age was associated with a decrease in mean lettuce consumption recorded over a 5 week period. In the young group lettuce consumption averaged 76.6 ± 3.5 g. Consumption was reduced in the middle-aged group to 64.3 ± 2.72 g and further reduced in the old group to 34.9 ± 2.79 g. ANOVA showed the three groups to be significantly different ($p < 0.001$), with both the middle aged and old groups being significantly different from the young group ($p < 0.05$ and <0.01, respectively; Tukey test). The old group also

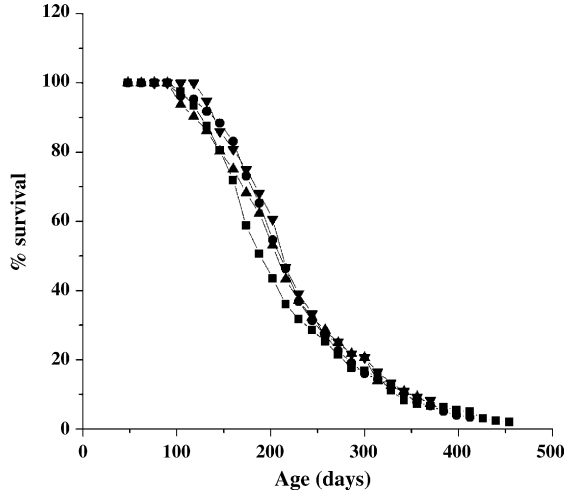

Fig. 2. Survival curves for populations of *Lymnaea* maintained in laboratory culture conditions. The graphs show the plots for four separate batches of *Lymnaea* maintained in four different tanks. Mean 50% survival occurs at 215.2 ± 10.9 days.

consumed significantly less lettuce than the middle-aged group ($p < 0.01$).

3.3. Short-term feeding experiments

In order to determine whether age affected the properties of the rhythm generating circuit a series of experiments were performed to examine the ability of sucrose to evoke short-term (2 min) feeding responses. In each of the three age groups approximately 4% of animals failed to emerge from their shells within 2 min of being placed in the test arena. Analysis of the % of non-emergers from each of the three age groups showed no significant age differences ($p > 0.05$, $n = 48$ per group). Of the animals that did emerge, there was a significant age-related increase in the % of animals that failed to respond to the standard sucrose stimulus. In the young group, 0.01 M sucrose stimulus was capable of evoking a feeding response in all animals tested. In the middle-aged age group, the number of non-responding animals increased to 7% and further increased in the old group to 12% ($p < 0.05$ between the young and old groups $n \geq 40$ per group).

In the remaining responding animals increasing age caused an increase in the duration of the inter-bite interval ($p < 0.001$; Fig. 3A) and a significant reduction in the number of sucrose-evoked bites ($p < 0.001$; Fig. 3B). The duration of the bite also increased significantly with age ($p < 0.05$; Fig. 3C). Mean bite durations for the young and middle-aged groups were not significantly different but both were significantly lower than times recorded in the old group (Fig. 3C). The emergence time and latency to first bite were not affected by age ($p > 0.05$ data not shown).

It was possible the changes detailed above were due to changes in the level of satiation or differences in the body weights of animals in the different age groups. However, control experiments showed that neither of these parameters significantly affected the overall age results.

3.4. Video analysis of feeding movements

It was not possible to analyze feeding responses from all the animals detailed in Section 3.3 as some moved from view of the camera while feeding. Animals were only used if the first 10 bites following sucrose application were visible to the camera and therefore, the data presented here represents a sub-set of the data described in Section 3.3. Increasing age was found to cause a significant increase in the duration of the swallow phase ($p < 0.01$, Fig. 4C), although these differences were only significant between the young and old groups. The duration of the inactive phase was also increased with increasing age ($p < 0.05$, Fig. 4D). Significant increases were also seen in the duration of the protraction phase of the cycle ($p < 0.01$, Fig. 4A), that mirrored the increases seen in bite duration (see Fig. 3C). There was no significant difference in the duration of the rasp phase of the cycle (Fig. 4B).

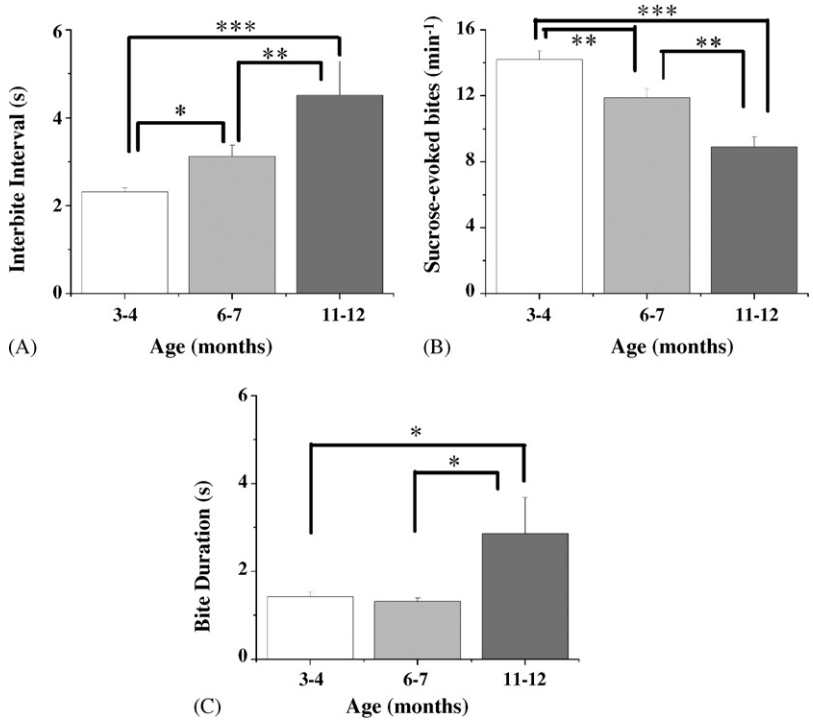

Fig. 3. Effects of increasing age on short-term feeding responses evoked by 0.01 M sucrose. Plots in A–C demonstrate changes in the inter-bite interval (A), number of sucrose-evoked bites min^{-1} (B) and bite duration (C). Values represent mean ± S.E.M. Significant differences were analysed using a one-way ANOVA followed by a post-hoc Tukey test (Minitab). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

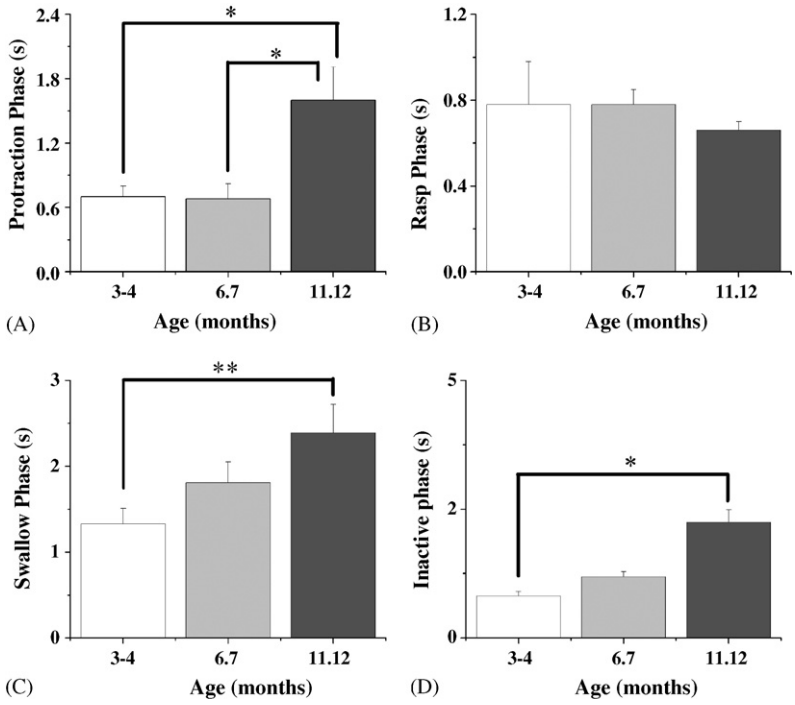

Fig. 4. The durations of the phases of each feeding cycle are altered with increasing age. A–D show plots of the changes in duration of the protraction (A), rasp (B), swallow (C) and inactive phase (D) of each feeding cycle with increasing age. Values represent the mean ± S.E.M. Significant differences were determined using an ANOVA followed by a post-hoc Tukey test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 12$ per group.

3.5. Effects of varying sucrose concentration on the feeding behavior of young and old animals

100% of young animals exposed to the 0.01 and 0.05 M sucrose stimuli responded with a feeding response, but there were a significant number of old animals that failed to respond to either of these two concentrations of sucrose. These differences were not seen at the highest sucrose concentration of 0.1 M (Fig. 5A). The number of sucrose-evoked bites were significantly greater in the young group compared to the old for both the 0.01 and 0.05 M sucrose concentrations, but not significantly different for animals exposed to 0.1 M sucrose (Fig. 5B). At both the 0.01 and 0.05 M sucrose concentrations, young animals had significantly shorter inter-bite intervals than the old group (Fig. 5C). Increasing the sucrose concentration to 0.1 M negated this difference.

3.6. Effects of increasing age on the chemosensory pathway

Concentration-dependent differences in the % of non-responding animals, number of sucrose-evoked bites and inter-bite interval in the young and old age groups suggested that there might be differences in the functioning of the chemosensory pathway between these two age groups. In order to test this possibility, extracellular recordings were made from the mln and the cbc in semi-intact preparations from young and old animals. Fig. 6A shows a typical recording from the mln and cbc of both a young and old snail. Activity in both the mln and cbc of the young and old animals was seen to increase during the 30 s application of 0.05 M sucrose and return to baseline when the sucrose perfusion was stopped. In Fig. 6B and C, the mean change in unit frequency of the mln and cbc is plotted versus time for animals exposed to the 0.01, 0.05 and 0.1 M sucrose concentrations. Increases in activity were seen in both the mln and cbc when all three sucrose concentrations were applied to the lips of both the young and old animals (Fig. 6B and C). Statistical analyses of the mean change in nerve frequency during the period of sucrose application for both nerves and for the three different sucrose concentrations showed no significant concentration effect (Fig. 6D). Analysis of the mln data showed no clear age effects, however significant age effects were seen in the cbc activity for animals exposed to both 0.01 and 0.05 M sucrose with the activity of the old cbc significantly lower than that seen in the young group ($p < 0.05$ for both groups). This significant difference was not observed in animals exposed to the 0.1 M sucrose stimulus (Fig. 6D).

4. Discussion

The experiments described in this paper provide an initial characterization of the effects of increasing age on the feeding behavior of the pond snail, *L. stagnalis* and the putative role that changes in the chemosensory pathway play in regulating these changes.

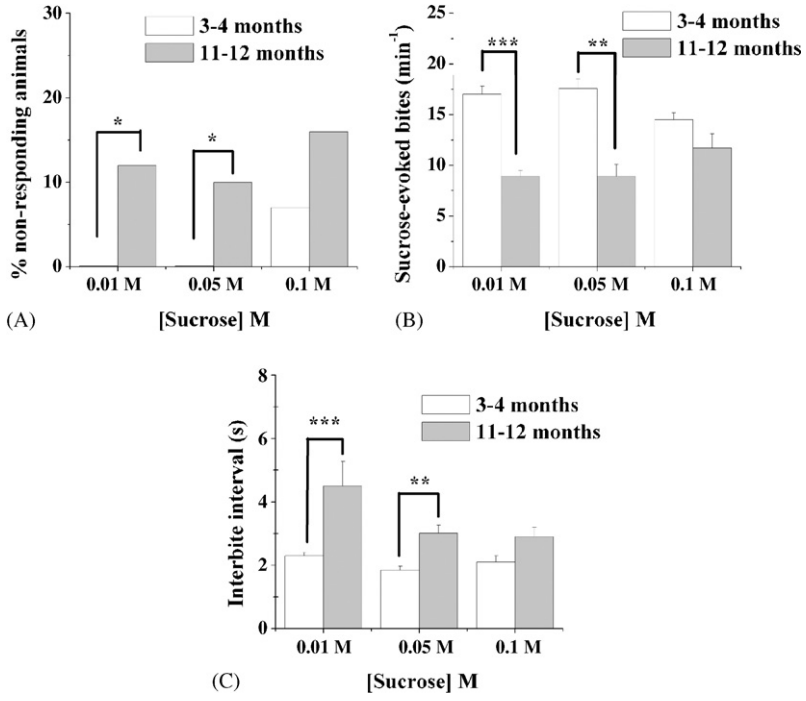

Fig. 5. Effects of age on the ability of different sucrose concentrations to activate feeding behavior. (A) At sucrose concentrations of 0.01 and 0.05 M the % of non-responding animals and in the 11–12 month group was significantly greater than the 3–4 month group. There was no significant difference age difference between groups of animals exposed to the 0.1 M sucrose stimulus. In those animals that did respond aged animals showed significantly fewer bites (B) and a longer inter-bite interval (C) than the young group at the two lower sucrose concentrations. At 0.1 M sucrose, no significant age effects were seen for either parameter. $n \geq 12$ per group, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4.1. Survival characteristics of L. stagnalis under laboratory conditions

The survival characteristics of four batches of *L. stagnalis* were studied under constant culture conditions. 50% survival varied from 6–8 months in the four populations with 15% survival occurring between 11 and 12 months. Maximum lifespans of animals maintained under these conditions was 15 months. The survival curves could all be fitted with a Weibull function and there were no significant differences between the survival curves indicating the reproducibility of the culture system. Although our mean and maximal survival times were less than previous studies in *Lymnaea* [19] these differences probably represent subtle changes in the feeding regimes between the two laboratories. Alterations in food intake have previously been shown to change lifespan in *Lymnaea* [20]. This probably represented a change in calorie intake a method that has previously been shown to alter lifespan in a wide variety of species ranging from *Drosophila* to mammals [2,20,35].

4.2. Age-related changes in feeding behavior

Lettuce consumption, measured over a 5 week period was seen to decline with increasing age, with the oldest animals consuming approximately 50% less lettuce compared to their young counterparts. These decreases may have been related to the decreases in both egg laying and growth which have previously been shown to occur around the 6–7 month age range [21]. Both these processes are energetically demanding and a decrease in both would therefore lead to a reduction in energy requirements. In mammals food intake has also been shown to decrease with increasing age although any marked decrease was limited to the last few weeks of life and is usually a good marker of imminent death [30]. This discrepancy probably represents differences in the thermoregulatory processes utilized by the two phyla, with mammals needing to generate their own heat which provides a constant metabolic demand, while *Lymnaea* is a poikilotherm.

Increasing age was also associated with a number of significant changes in short-term sucrose-evoked feeding responses. Clear increases were seen in the number of animals that failed to respond to the sucrose stimulus as age increased. Of the animals that did respond increasing age was associated with an increase the duration of the swallow phase of each feeding cycle and a reduction in the number of sucrose-evoked bites. The degree of change was age-dependent and was consistently seen in all batches of animals tested. Increases were also seen in the duration of the bite, although statistically significant changes were less consistent between different batches of animals. Additionally, the observed increases were only seen in the old group, indicating a process distinct from the one regulating the swallow phase and number of sucrose-evoked bites. Previous work in rats has shown that increasing age is associated with decreases in the rate of food consumption [8] and a decrease in licking

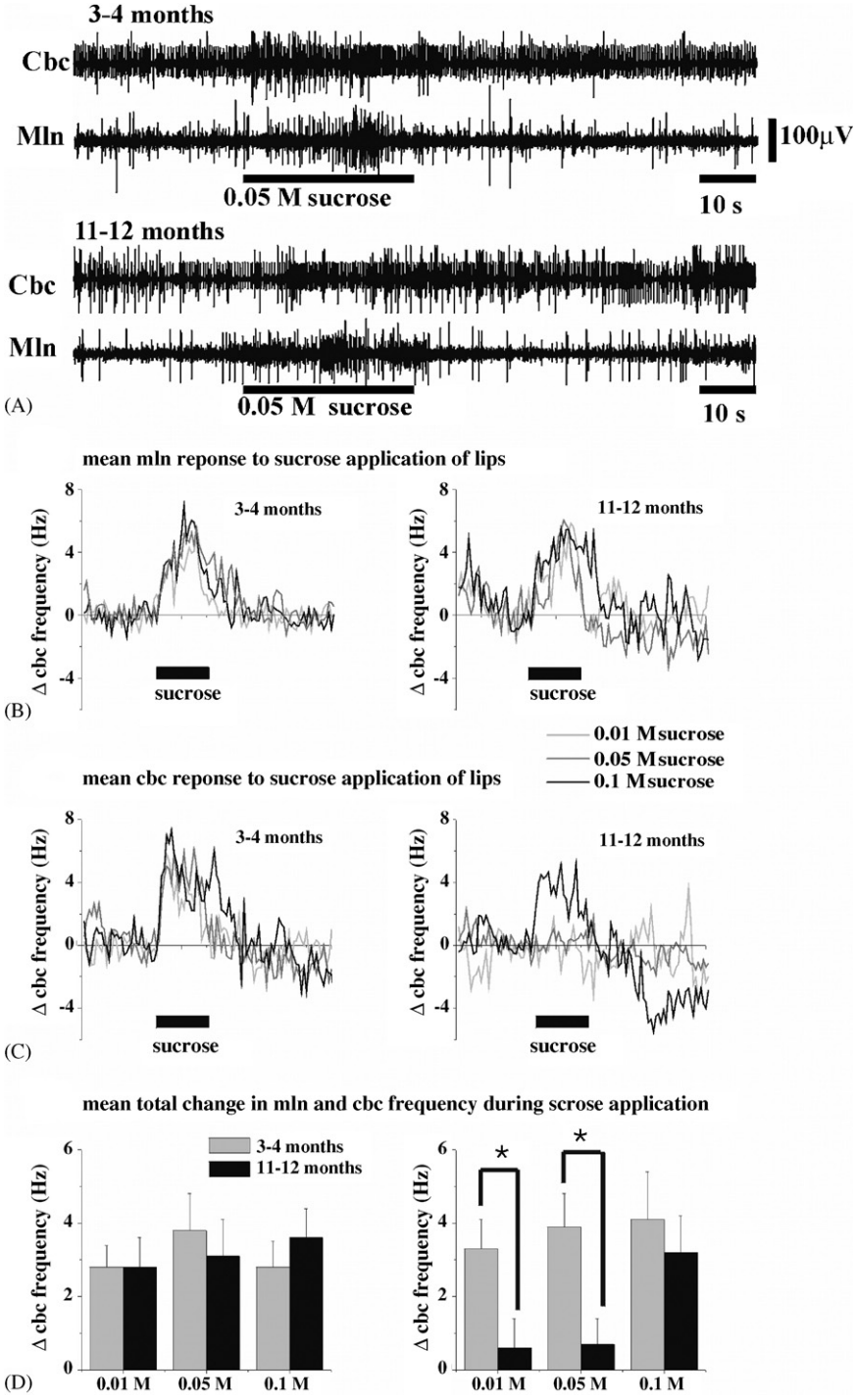

Fig. 6. Increasing age affects central processing of the chemosensory signal. (A) Typical extracellular recordings from the cerebrobuccal connective (cbc) and a median lip nerve (mln) in a semi-intact preparation consisting of the lips and CNS of a young and old snail. Application of 0.05 M sucrose to the lips caused a marked increase in the electrical activity recorded in both the mln and cbc of both animals. (B and C) Quantitative analysis of the change in activity of the mln (B) and cbc (C) in response to application of a range of sucrose concentrations in both young and old animals. Electrical activity in the nerves was determined by dividing the data into 2 s bins. The count in each bin was baseline corrected by subtracting the average number of events for the 15 bins prior to stimulus application from each bin. The baseline subtracted data for equivalent experiments was averaged and plotted giving the time course of the net average change in nerve activity in 2 s bins for each stimulus. There were no differences in the firing frequency of the mln from both young and old animals in response to three sucrose applications tested. Responses of the cbc were reduced in the old animals compared to the young at 0.01 and 0.05 M sucrose concentrations but were not obviously different in animals exposed to 0.1 M sucrose. (D) Bar graph to show how the average changes in both mln and cbc activity during the period of sucrose, vary with age. No clear age effects were seen in the activity of the mln, however recordings from the cbc showed that young animals had consistently higher changes in activity than the old animals across the whole range of sucrose concentrations tested although these were only significant at 0.01 and 0.05 M sucrose concentrations. Values in D represent mean ± S.E.M., $^{*}p < 0.05$, $n \geq 13$ for each group.

frequency [42] another CPG-driven behavior, and work in humans by a number of different groups has shown clear age-related increases in the duration of swallowing [36,41]. These data are therefore suggestive that the effects of increasing age on the regulation of feeding are common across a wide range of phyla.

Video analysis of the results from these short-term feeding experiments demonstrated that the increases seen in the bite duration with increasing age were due solely to increases in the N1 (protraction) phase of the feeding cycle with no significant change in the duration of the N2 (rasp) phase. The duration of the swallow phase in the behavioral experiments comprised the N3 (swallow) phase plus a component of the inactive phase that divided successive feeding cycles. Video analysis allowed these two phases of the feeding cycle to be separated and demonstrated that the increase was due to a prolongation of both phases of the cycle. The duration of the inactive phase is determined by the speed of transition from the N3 (swallow) phase to the N1 (protraction) phase [47]. Its duration has previously been shown to be inversely proportional to the frequency of the feeding rhythm [4] and therefore its observed increase in aged animals may be a consequence of the prolonged N1 (protraction) and N3 (swallow) phases of the feeding cycle.

4.3. Role of changes in the chemosensory pathway

Significant age-related deficits were seen in the % non-responding animals, the inter-bite interval and the number of sucrose-evoked bites of animals exposed to either the 0.01 or 0.05 M sucrose solutions. However, increasing the concentration of sucrose to 0.1 M negated these differences. Interestingly, previous work in *Aplysia* has demonstrated that it is possible to overcome age-related deficits in the ability of the animals to sense external stimuli by increasing the strength of the stimulus [34]. The differential effects of sucrose on the two age groups suggested that age-related changes in the ability of the chemosensory pathway to activate feeding may contribute to the short-term feeding deficits seen with increasing age. These changes may represent alterations in the sensitivity of the primary sensory neurons in the lips or may be reflective of changes elsewhere in the chemosensory pathway. Results from experiments in which the electrical activity of the median lip nerve was recorded following application of sucrose to the lips demonstrated that there was no significant effect of age on the response patterns of the nerve to a range of sucrose concentrations. This indicated that there was no age-related change in the frequency of information passing from the primary sensory neurons in the lips to the cerebral ganglia. It was however possible that the signals recorded in the young and old nerves represented activity from different populations of sensory neurons. However, as discussed by Straub et al. [45], for the net activity in the median lip nerve not to be altered would require a precise balancing of the changes in the activities of the different populations of units that has not been described to date in any sensory system. Clear decreases were however seen in the neural activity recorded in the cbc of the old compared to the young animals at the two lower sucrose concentrations, suggesting that processing of the chemosensory stimulus in the cerebral ganglia was altered with increasing age. The concentration-dependent age effects on cbc activity were seen to mirror the age-related changes in feeding behavior evoked by the same range of sucrose concentrations, suggesting that the changes in cbc activity maybe responsible for the age-related changes in feeding behavior. To our knowledge, the effects of ageing on chemoreception has not previously been studied in other mollusks and therefore whether changes in central processing are common to all mollusks is unclear. Similar changes in central rather than peripheral processing were seen in *Lymnaea* in animals that had been appetitively conditioned to respond to amyl acetate were compared to naïve animals [45], suggesting that the cerebral ganglia are an important site for neuronal plasticity. Similar learning and memory studies in terrestrial mollusks have demonstrated changes in central processing of chemosensory information indicating that these sites of plasticity are present in other mollusks [15,23]. Work on the effects of age on chemoreception in rats showed a similar lack of change of the evoked electrical activity of the chorda tympani in response to a variety of different tastes [33], suggesting that peripheral processing of chemosensory information in this species is also not affected by age.

4.4. A neurophysiological correlate for age-related changes in feeding behavior

The work described in this paper demonstrates that aging is associated with clear alterations in the sucrose-evoked feeding response. Specifically, increasing age caused a significant increase in the % non-responding animals, the bite duration, and inter-bite interval and a marked decrease in the number of sucrose-evoked bites. Previous work in *Lymnaea* has documented that the CGCs are incapable of initiating a feeding rhythm but have an important modulatory role allowing the feeding CPG to respond to a food stimulus (gating role) and can also regulate the frequency of fictive-feeding [frequency controlling role; 49,51]. Thus deficits in the functioning of the CGCs could account for both the age-related changes in the number of non-responding animals and the decrease in sucrose-evoked bites. Additional support for the CGCs being a neurophysiological correlate of aging comes from work by Kemenes et al. [24] who examined short-term feeding responses in *Lymnaea* that had been pre-treated with the serotonergic neurotoxin, 5,6-dihydroxytryptamine (5,6-DHT). Serotonin (5-HT) is the main transmitter utilized by the CGCs and therefore injection of the toxin would lead to either a complete or partial loss of CGC function. Their results demonstrated that 5,6-DHT caused a marked increase in the number of non-responding animals, an increase in the inter-bite interval and a decrease in the number of sucrose-evoked bites and were consistent with the CGCs being a target

for the effects of age. Work in a closely related mollusk, *Aplysia*, demonstrated that destruction of the metacerebral cells (MCCs), homologs of the CGCs also caused similar deceases in the frequency of biting movements and a decrease in the inter-bite interval further substantiating the role of the CGCs in the aging of this network [39].

The data presented in this paper infers that a site in the cerebral ganglia is responsible for processing the sensory information and that this site is malfunctioning in the aged animals. The CGCs of *Lymnaea* have previously been shown to be involved in the processing of sensory information. Work in this species has shown that application of sucrose to the lips of the animal induces a burst of action potentials in the CGCs in semi-intact lip-CNS preparation [22,31]. A similar increase in activity was seen in *Lymnaea* in response to the application of lettuce to the lips in the intact animal [51]. In *Aplysia*, a closely related mollusk, application of a feeding stimulus, seaweed, to the lips of the animal induced a burst of action potentials in their homologs, the metacerebral giant cells [18,25].

In summary, the data presented in this paper demonstrate that increasing age is associated with a deficit in the sucrose-evoked feeding responses. Using our prior knowledge of the regulation of the feeding system it appears that the ability of the feeding CPG to generate a basic three phase rhythm is not affected by increasing age. Varying the sucrose concentration used to evoke feeding yielded specific concentration-dependent effects of age on the feeding behavior of young and old animals. These changes were mirrored by concentration-dependent changes in cbc but not mln activity. This suggested that alterations in the central, but not peripheral processing of the chemosensory information contributed to the age-related changes in feeding behavior. It is proposed that these changes are likely to involve a deficit in the functioning of the modulatory serotonergic CGCs.

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