Author(s): Waselius, Tomi; Wikgren, Jan; Penttonen, Markku; Nokia, Miriam

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Year: 2019

Version: Accepted version (Final draft)

Please cite the original version:

Waselius, T., Wikgren, J., Penttonen, M., & Nokia, M. (2019). Breathe out and learn : Expiration-contingent stimulus presentation facilitates associative learning in trace eyeblink conditioning. Psychophysiology, 56(9), Article e13387. https://doi.org/10.1111/psyp.13387

Breathe out and learn: expiration-contingent stimulus presentation facilitates associative learning in trace eyeblink conditioning

Tomi Waselius[1]*, Jan Wikgren[1,2], Markku Penttonen[1] & Miriam S. Nokia[13]

[1]Department of Psychology, P.O. Box 35, 40014 University of Jyvaskyla, Finland

[2]Centre for Interdisciplinary Brain Research, University of Jyvaskyla, Finland

[3]Neuroscience Center, Helsinki Institute of Life Science, University of Helsinki, Finland

*corresponding author: tomi.waselius@jyu.fi

ABSTRACT

Rhythmic variation in heart rate and respiratory pattern are coupled in a way that optimizes the level of oxygen in the bloodstreams of the lungs and the body as well as saves energy in pulmonary gas exchange. It has been suggested that the cardiac cycle and respiratory pattern are coupled to neural oscillations of the brain. Yet studies on how this rhythmic coupling is related to behavior are scarce. There is some evidence that, for example, the phase of respiration affects memory retrieval and the electrophysiological oscillatory state of the limbic system. It is also known that the phase of the cardiac cycle and hippocampal electrophysiological oscillations alone affect learning. Here we studied whether the timing of training trials to different phases of respiration affects learning trace eyeblink conditioning in healthy adult humans. Trials consisting of a neutral conditioned stimulus (200-ms tone) and a slightly aversive unconditioned stimulus (100-ms airpuff towards the eye), presented with a 600-ms trace interval, were timed to either inspiration or expiration. A control group was trained regardless of respiratory phase. We found that, at the end of training, the rate of conditioned responses was higher in the group trained at expiration than it was in the other two groups. That is, brain state seems to fluctuate as a function of respiratory rhythm and this fluctuation is also behaviorally relevant, exerting its effect on, at the least, a simple form of associative learning.

Keywords: memory, respiration, respiratory sinus arrhythmia

1. INTRODUCTION

The effect of rhythmic variation of bodily functions on brain oscillations and cognition is an emerging field of research. Since the 1950s, it has been known that respiratory pattern and cardiac cycle are synchronized, a phenomenon termed *respiratory sinus arrhythmia* (RSA) (Bregher & Hubay, 1955). In short, the cardiac cycle is longer during expiration (breathing out) and shorter during inspiration (breathing in) (Berntson, Cacioppo, & Quigley, 1993; Hirsch & Bishop, 1981; Katona & Jih, 1975). One function of RSA in vertebrates is to optimize gas exchange in the lungs and periphery (Yasuma & Hayano, 2004). This cardiorespiratory regulation is driven by the pons of the brainstem via baroreceptors located on the walls of major blood vessels (Farmer, Dutschmann, Paton, Pickering, & McAllen, 2016). It is known that the phase of the cardiac cycle alone affects neural processing of external stimuli in humans (Gray et al., 2012; Martins, McIntyre, & Ring, 2014; Park, Correia, Ducorps, & Tallon-Baudry, 2014; Waselius, Wikgren, Halkola, Penttonen, & Nokia, 2018). For example, negative pictures are judged to be more intense when presented during systole (Gray et al., 2012). In addition, in rabbits, learning trace eyeblink conditioning (TEBC), a hippocampus-dependent variant of classical Pavlovian conditioning (Holland & Bouton, 1999; Solomon, Vander Schaaf, Thompson, & Weisz, 1986), is most effective if the conditioned stimulus is timed to the diastolic phase of the cardiac cycle (Waselius, Wikgren, Halkola, Penttonen, & Nokia, 2018). Thus, rhythmic variation in bodily functions seems to have an impact on the encoding of external information in the brain.

Bodily functions, and especially brain activity, show rhythmic variation or, in other words, they oscillate. It has been suggested that the electrophysiological activity of the brain is organized based on harmonic frequencies, and that the different oscillations serve different physiological functions (Penttonen & Buzsáki, 2003). For example, 3–12 Hz theta oscillations emerge in the mammalian hippocampus during exploratory activities (Buzsáki, 2002) and in part regulate the firing of pyramidal cells that encode the location of the subject, so-called place cells (J O'Keefe & Dostrovsky, 1971; John O'Keefe, 1976). In addition, gamma frequency (30–80 Hz) in the hippocampus is phase locked to theta during maze exploration and rapid eye movement sleep (Belluscio, Mizuseki, Schmidt, Kempter, & Buzsáki, 2012). Together, the theta and gamma oscillations pace activity of hippocampal cells: for example, hippocampal interneurons fire action potentials phase-locked to both frequencies (Bragin et al., 1995). Sirota et al. (2008) suggest this kind of synchronization ensures that the timing of information processing is optimal for plasticity.

In addition to this harmonic coupling within rhythms of the brain, neural oscillations are also coupled to those of the body (Klimesch, 2013, 2018). For example, breathing rhythm is connected to oscillatory brain activity in widespread brain regions (for a review, see Tort, Brankačk, & Draguhn, 2018). More specifically, using depth electrodes in human epilepsy patients, it was found that the power of electrophysiological oscillations in the delta (0.5–4 Hz), theta (4–8 Hz) and beta (13–30 Hz) frequency bands in the piriform cortex, the amygdala and the hippocampus increases during inspiration. Interestingly, this phase-amplitude coupling between respiration and limbic system electrophysiological activity was specific to nasal breathing. Similar observations have also been made in mice (Tort, et al., 2018; Yanovsky, Ciatipis, Draguhn, Tort, & Brankačk, 2014). For example, in urethane-anesthetized mice the olfactory bulb and the hippocampus exhibit respiration entrained oscillations distinct from spontaneous hippocampal theta activity (Yanovsky et al. 2014). Furthermore, oscillatory activity (2-14 Hz) in the hippocampus (and other brain areas) follows breathing rhythm and is coherent with the hippocampal theta oscillation during exploratory activity (Tort et al. 2018b). However, these oscillations also occur incoherently with the theta oscillation. Simply put, respiration-driven neural activity in the hippocampus is often distinct from hippocampal theta (Tort et al. 2018b).

In addition to theta and gamma, electrophysiological oscillations typical for the limbic system also include the so-called sharp wave-ripples (SPW-R). These are fast bursts of hippocampal neural activity (110–200 Hz) that are present usually during sleep and immobility (Buzsáki, 1986; John O'Keefe, 1976). SPW-Rs represent synchronous excitation of CA1 pyramidal neurons (Buzsáki, 1986) and are thought to reflect memory consolidation (for a review, see Buzsáki, 2015). However, SPW-Rs also occur while awake and, in fact, presenting conditioning stimuli contingent on SPW-Rs enhances learning of TEBC in rabbits (Nokia, Penttonen, & Wikgren, 2010). Interestingly, SPW-Rs more likely occur during expiration in mice (Liu, McAfee, & Heck, 2017) and, even more curiously, this SPW-R modulation is dependent on an intact olfactory bulb.

Taken together, inspiration seems to promote rhythmic activity at 0.5–30 Hz in the limbic system (Zelano et al., 2016) while SPW-Rs are more likely to occur in the hippocampus during expiration (Liu et al., 2017). Because previous results also indicate that training contingent on SPW-Rs is beneficial to learning (Nokia et al., 2010), we suggest that the overall neural state could be more favorable for acquisition of new information during expiration than it is during inspiration. To our knowledge, the direct effects of respiratory rhythm on associative learning have not been studied. In this experiment, we trained healthy adult participants in TEBC. In two experimental groups, the training trials consisting of a conditioned stimulus (CS) followed by an unconditioned stimulus (US) were timed either at the inspiration or the expiration phase. A control group was trained at random, irrespective of respiratory rhythm. As explained above, we hypothesized that participants trained at the expiration phase would learn better than those trained at random or at the inspiration phase.

2. METHOD

2.1 Participants

The participants in this study were recruited via e-mail lists. They gave informed written consent to this study and were free to discontinue participation in the experiment at any point knowing that they would still get a reward (a movie ticket) by participating in the experiment. The study was approved by the University of Jyväskylä Ethical Committee. Thirty-six adults (12 males; aged 20–30 years: mean 24.1) took part in the study. Two of the participants were left-handed. All of the participants were healthy with no history of psychiatric or neurological illnesses. They were not taking medication affecting brain function, and had no disability in hearing or vision.

Experimental procedure

The schematic structure of the experimental procedure is presented in Figure 1.

2.2.1 Recordings

Recording electrodes were attached after participants had signed the written consent. Respiration was recorded and monitored during the experiment with a reusable fabric belt (RESPA00000, Spes Medica, Italy), which was fastened on top of the clothes on the lower chest area. Heart rate was recorded using three electrocardiogram (ECG) electrodes (Kendall, H92SG); one electrode was placed on top of the right clavicle, one on the left lower ribs, and the grounding electrode on the back of the neck. Eyeblinks (see Figure 2B) were recorded using two electrodes (70010-K/12, Ambu, Ballerup, Denmark) that were attached on top of the participant’s right eye muscles (orbicularis oculi). All signals were high-pass filtered (0.16 Hz) and low-pass filtered (250 Hz) online and recorded with NeurOne Tesla (with Analog Out Option, Bittium Biosignals Ltd., Finland).

2.2.2 Instructions to participants before conditioning

The participants sat in a chair in front of a TV screen (Asus VG236 series H, 23”; distance: ~100 cm). They were informed that the aim of the study was to record physiological responses to different types of stimuli while their attention was to be directed at a silent film depicting landscapes and animals. The participants were instructed to follow the film, because there would be questions considering the content of the footage after the session. They were also instructed to sit comfortably in the chair and not pay attention to the disturbing stimuli. In other words, the participants were led to believe that the idea was to study the disturbance caused by beeping sounds and air puffs on their attention towards the film.

2.2.3 Trace eyeblink conditioning

The conditioned stimulus (CS) was a 200-ms, 440-Hz, 66-dB tone delivered via a loudspeaker situated in the lower right-hand corner of the room. The unconditioned stimulus (US) was an airpuff (0.2 bar source pressure, 100 ms) targeted at the right eye and it was delivered via a plastic tube attached to modified safety goggles. Note that the air pressure was low and none of the participants felt that the air coming to the eye was unbearable. During conditioning trials, a 600-ms trace interval separated the tone offset and the US onset. The presentation of stimuli used for conditioning was controlled by custom software running on an Arduino-based device (ABD).

First, four US-alone trials with an inter-trial interval (ITI) of 5 s were presented to make sure that the participants felt comfortable enough to proceed with the experiment. After this, 2 minutes of resting data were recorded, followed by five CS-alone trials (random ITI 20-40 s). Then, 50 CS+US conditioning trials (ITI 20-40 s) were presented either at inspiration, expiration or at a random phase of breathing.

To this end, the respiration and EMG signals were conveyed to a custom script running in LabVIEW (National Instruments). Both signals were sampled at 1 kHz. At each time point, the last second of both EMG and respiration signals were analyzed. EMG was evaluated for spontaneous eyeblinks, that is, the signal had to stay below a set amplitude threshold in order to proceed with presenting the conditioning trials. The respiration signal was analyzed in two consecutive 500-ms windows. In order to trigger a trial, the signal amplitude during the latter 500-ms time window had to cross a set absolute threshold value (peak for inspiration, trough for expiration) and the signal had to either rise (inspiration) or fall (expiration) at a certain rate between the two consecutive time windows. Note that the threshold values for the EMG and for the respiration peak and rise (inspiration) and for the trough and fall (expiration) were set individually for each participant during the 2-minute baseline recording prior to conditioning. As an end result, when the participant was not spontaneously blinking and respiration was at a desired phase, LabVIEW sent a TTL pulse to the ABD, which then presented the actual conditioning stimuli (see above).

Two minutes of spontaneous breathing without any external stimuli were recorded after the conditioning to visually confirm on-line that the experimental manipulation had not changed the respiration pattern overall and that the respiration belt signal quality had remained similar to that recorded before experimental manipulations. The whole procedure lasted about 40 minutes depending on the random ITI.

2.2.4 Questions to participants after conditioning

After the experiment, participants answered background questions about age, gender, and handedness and five questions concerning the silent film (e.g., “What kind of footwear did the man use in the snow at the beginning of the film?”). One open question about the disruptive stimuli was also presented: “When did the airpuff occur?”

2.3 Data analysis

2.3.1 Conditioned responses

The percentage of conditioned responses (CR) performed by each participant was analyzed off-line using MATLAB (The MathWorks Inc.).The signal was low-pass filtered (40 Hz) and the absolute values for the signal were calculated. The mean amplitude during a 500-ms pre-US period (MEANpre) was calculated. In addition, the mean of the standard deviation of the amplitude during the 500-ms pre-CS period (SDpre) was determined. Learned responses were detected from a 200-ms time window immediately preceding the US. To qualify as a learned response, the eye muscle activity had to exceed the following threshold: MEANpre + 2*SDpre. Trials were grouped into five blocks of 10 trials (CC1, CC2, CC3, CC4 and CC5) for analysis and the percentage of learned responses per block was calculated.

2.3.2 Respiration, ECG and the respiratory sinus arrhythmia (RSA)

RSA was analyzed off-line using MATLAB by searching epochs of respiratory phases (inspiration and expiration) with at least two consecutive R-peaks within a phase and calculating the mean interval (in ms) between these R peaks. Epochs with only one R-peak were rejected from the analysis (Grossman, Beek, & Wientjes, 1990).

2.3.3 Statistics

A paired samples *t* test was used to compare R-peaks during inspiration vs. expiration. Repeated-measures analysis of variance (ANOVA), with five training blocks of 10 trial averages each as a within-subjects factor and group (3) as a between-subject factor, was used to analyze changes across training and differences between experimental groups in learned responding. In case the assumption of sphericity was violated, *p* values were corrected using Huynh-Feldt estimates. A one-way ANOVA was used for comparisons between groups at each trial block. For post hoc comparisons, Bonferroni-corrected *p* values are reported.

3. RESULTS

3.1 R-peak intervals were longer during expiration

To first verify the existence of RSA in our current data we analyzed the interval between R-peaks at inspiration and expiration. A paired samples *t* test showed that there was a statistically significant difference in R-peak interval (in milliseconds) between inspiration and expiration throughout the experiment (in all participants): The interval between R-peaks was longer during expiration (mean ± standard deviation: 764 ± 83 ms) compared to inspiration (737 ± 78 ms); t (35) = 4.96, $p < 0.0001$, $d = 0.836$. In other words, the heart rate was slower during expiration and the overall heart rate was around 80 beats per minute.

3.2 Timing of trials to expiration enhanced learning

Next, we studied the effects of TEBC (5 training blocks) and respiration phase (Inspiration, Expiration, Random) on learned responding (see Figure 3) using a repeated measures ANOVA. There was a significant interaction between group and training block: F (8, 132) = 2.478, $p = 0.019$, $p\eta^2 = 0.14$ [main effect of training block: F (4, 132) = 8.413, $p < 0.0001$, but the main effect of group: F (2, 33) = 0.522, $p = 0.600$] was not significant. These results suggest learning curves in the three groups were different. Further analysis of the significant group and training block interactions showed that the effect of training on learned responding was significant in the Expiration group [F (4, 44) = 5.182, $p = 0.002$] and in the Random group [F (4, 44) = 5.681, $p = 0.001$] but not in the Inspiration group [F (4, 44) = 2.248, $p = 0.079$].

To further analyze at which stage of the learning process the groups differed from each other, separate one-way ANOVAs were conducted at each trial block. This analysis did not reveal a significant difference in the CR percentage until the last (5th) training block [F (2, 35) =

4.275, *p* = 0.022, η² = 0.26; blocks 1 to 4: *F* (2, 35) = 0.92 – 1.282, *p* = 0.291 – 0.912]. The CR percentage (%) during the 5th training block was highest in the Expiration group (73.33 ± 30.55) and lower in the Inspiration (45.0 ± 28.44) and Random (47.5 ± 18.15) groups. Post hoc comparisons (Bonferroni) indicated that the difference was significant between the Expiration and Inspiration groups: *p* = 0.038. The difference was also very close to significance between the Expiration and Control groups: *p* = 0.065. There was no difference in CR percentage between the Control and Inspiration groups: *p* = 1.00. To conclude, the three groups acquired the CR at different rates, and this difference is due to the Expiration group performing more CRs than the other two groups at the end of conditioning.

3.3 Participants paid attention to their primary task but also became aware of the stimulus contingency

All of the participants were instructed to pay attention to a silent film while ignoring other stimuli. Of the 36 participants, 35 were able to answer close to 100% correctly on the questionnaire considering the landscape footage and animals (only one wrong answer from 180 answers). However, all of the participants also correctly answered the open question about the TEBC stimulus presentation (“When did the airpuff occur?”), stating, for example, “At the beginning, there were a few tones alone and after a while a tone followed by an airpuff started to occur” or “The tone was always followed by an airpuff.” In other words, the participants became aware of the CS-US contingency even though they had been concentrating on the film.

4. DISCUSSION

We studied whether human participants concentrating on a silent film would learn TEBC better if the conditioning trial was presented at the expiration phase of respiration than if it was presented at the inspiration phase or at a random phase. All of the participants showed consistent RSA throughout the experiment, which indicates stability of the autonomic nervous system function. As a result of conditioning, all participants became aware of the CS-US contingency. At the end of the training, the participants explicitly trained during expiration performed more learned responses than those trained during inspiration. This suggests that the respiration phase during training has an effect on the outcome of TEBC.

Our results can be interpreted in the light of Prokasy's (1987) theory, which states that learning proceeds in two stages. In the first phase an association is formed between the CS and the US and the appropriate CR is selected. In the second phase, the likelihood of performing a CR increases and the CR is adjusted to maximize the adaptivity of the response. It seems that in our current study the experimental manipulation affected mostly the latter phase of learning. That is, once acquired, the performance of the adaptive learned response was more likely during expiration than during inspiration. However, there was no significant difference in learning between subjects trained at random and either of the experimental groups. The fact that TEBC conducted irrespective of respiration phase resulted in learning to a degree that falls between the two experimental groups can be interpreted to further underline the different effects of inspiration and expiration phases of respiration on learned responding.

Our current results support the idea that bodily rhythms such as breathing and heart rate regulate cognition, possibly via their connection to rhythms of the brain. Recent studies indicate respiration-driven slow electrophysiological oscillations in the hippocampus

(Nguyen Chi et al., 2016; Tort, et al., 2018; Zelano et al., 2016). In addition, a study in mice reported that SPW-Rs (Buzsáki, 2015), the fast bursts of activity thought to mediate memory consolidation into the neocortex, occur in the hippocampus more likely during expiration, but only when the olfactory bulb is intact (Liu et al., 2017). The mechanisms behind these respiration-related hippocampal rhythms are still unclear. However, it is known that the rhythm of respiration is driven by the preBötzinger complex (preBötC) in the ventrolateral medulla of the brainstem (Pilowsky & Feldman, 2001; Smith, Ellenberger, Ballanyi, Richter, & Feldman, 1991). The cardiovascular function is also controlled by cells in the ventrolateral medulla (Brown & Guyenet, 1985). Physiologically and anatomically, the neurons pacing the respiratory and cardiovascular function are intertwined (Dergacheva, Griffioen, Neff, & Mendelowitz, 2010) and could have similar effects on the overall neural state. Interestingly, activation of Cdh9/Dbx1 neurons in the preBötC seems to evoke behavioral arousal whereas genetic ablation of Cdh9/Dbx1 neurons has opposite effects (Yackle et al., 2017). It has been suggested that activation of large neural populations in the preBötC also affects the overall state of the hippocampus and other brain regions (for a review, see Del Negro, Funk, & Feldman, 2018). Thus, it could be that both bodily and brain rhythms are paced by common orchestrators in the brainstem.

It is likely that there are bidirectional connections between the limbic system and the cardiorespiratory system. At any given time, the cardiorespiratory state is the outcome of the net balance between the parasympathetic and sympathetic nervous system. Yet through which mechanisms exactly these processes connect with the changing state of the limbic system remains unclear. Although the activation of the sympathetic–parasympathetic nervous system can modulate the RSA, it has been stated that respiration rate and tidal volume are only affected during, for example, repetitive mental tasks and aerobic exercise, but not in a steady state when an autonomic tone is constant (Grossman & Taylor, 2007; Grossman & Wientjes, 2001). This means that, in principle, in our current experiment, watching the silent film or the TEBC itself could have affected the state of the autonomic nervous system and thus respiration. However, according to our results, participants had a high level of RSA during the task, which indicates a constant state of the autonomic nervous system.

Our current results add to findings from our previous study in which TEBC was enhanced when the conditioned stimulus was timed to the diastolic phase of the cardiac cycle (Waselius et al., 2018). Our previous results (Waselius et al., 2018) suggest that the brain processes information differently during the diastolic and systolic phases. In short, responses to external stimuli are attenuated during the diastolic phase and amplified during the systolic phase. It could be that the large populations of neurons that drive the cardiovascular pace in the brainstem are less active during the diastolic phase of the cardiac cycle. It is very plausible that these neurons should drive the systolic phase, when the heart contracts. This could lead to a state of the nervous system that is more favorable for plasticity, such as enhanced SPW-R activity in the hippocampus (Buzsáki, 1989; Nokia et al., 2010). In further studies it would be interesting to study how respiration phase (or other bodily rhythms) affects for example neural activity in the amygdala, which also seems to play a significant role in TEBC (Büchel, Dolan, Armony, & Friston, 1999; Chau & Galvez, 2012).

When taken as a whole, our results indicate that the state of the brain could be even more optimal for learning at the diastolic phase of the cardiac cycle during expiration (Figure 4). Monitoring both respiration and the cardiac cycle could be useful when trying to facilitate learning.

4.1 Limitations

First, the sample size in this study is rather small, even though it is comparable with norms in the field. Second, recent studies have shown that nasal breathing vs. oral breathing have different effects on the limbic system (Zelano et al., 2016). While we did not explicitly instruct the participants to breathe only through the nose, we excluded participants with respiratory health problems such as the common cold. Furthermore, the experimenter monitored the recording via video camera throughout the conditioning session. In cases of excessive mouth breathing such as yawning, the experiment would have been discontinued. However, we observed no such activity during the experiment.

ACKNOWLEDGEMENTS

We thank Lauri Viljanto and Petri Kinnunen for their efforts in technical help in building the recording systems. We also thank Professor Heikki Tanila for his valuable comments. The work was supported by the Academy of Finland [grant number 286384 to M.S.N.].

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FIGURE LEGENDS

Figure 1 Experimental design

The participants were instructed to concentrate on watching a silent film. First, four air puffs later used as the unconditioned stimulus (US) were presented. After this, two minutes of resting data was recorded followed by five presentations of the tone-conditioned stimulus (CS) alone. Then, 50 CS+US conditioning trials were presented either at inspiration, expiration or a random phase of breathing. The trace period was 600 ms and the inter-trial interval varied between 20 and 40 s. Two minutes of data were recorded also after the conditioning procedure.

Figure 2. Conditioning procedure and an example of a learned response

A) Conditioned stimulus (CS) and unconditioned stimulus (US) were both timed either at inspiration, expiration (presented in the same figure) or at a random phase of respiration (Resp). B) Participants were trained in trace eyeblink conditioning (TEBC) with a 200-ms, 440-Hz, 66-dB tone as a CS and a 100-ms air puff as an US. The trace period between CS and US was 600 ms. A typical well-timed learned eyeblink response observed from one person during one trial is illustrated.

Figure 3. Participants trained at the expiration phase of respiration made more conditioned responses at the end of the conditioning procedure compared to those trained at the inspiration phase.

Blocks from CC1 to CC5 represent groups of 10 trials. There was insignificant spontaneous blinking during tone-alone trials. Learning in the Expiration group was linear whereas in the Inspiration and Random groups it was not. The Expiration group also performed better at the end of the training compared to the Inspiration group. Error bars equal to standard error of mean. Asterisk refers to one-way ANOVA, indicating statistically significant difference, Bonferroni-corrected post-hoc comparisons, $p < 0.05$.

Figure 4. The large population activation in the preBötzinger complex might project to the limbic system and therefore affect learning

We suggest that the brain is in a more favorable neural state for learning during the diastolic phase of the cardiac cycle especially during the expiration phase of respiration. The lighter the area is, the more favorable the timing is for plasticity, according to our suggestion. Continuous line = raw ECG, dotted line = respiration.