Aus dem

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# The effect of sleep on classical conditioning in *Aplysia californica*

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Meiner Familie

# I. Table of content

**I. Table of content** .... **III**

**II. List of figures and tables** .... **VI**

**III. List of abbreviations** .... **IX**

**1. Introduction** .... **1**

1.1. Learning and memory .... 1

1.1.1. Learning .... 1

1.1.2. Memory .... 2

1.2. Sleep .... 4

1.2.1. Physiology of sleep .... 4

1.2.2. The function of sleep .... 6

1.2.3. Sleep's role for memory consolidation .... 8

1.3. *Aplysia* as a model for sleep and memory .... 10

1.3.1. Anatomy of *Aplysia* .... 10

1.3.2. Chemical defense .... 11

1.3.3. Learning paradigms in *Aplysia* .... 12

1.3.4. Sleep deprivation in *Aplysia* .... 15

1.4. Previous work on conditioning memory and sleep in *Aplysia* .... 17

1.5. Summary and aim of this work .... 19

**2. Material and Methods** .... **21**

2.1. Animal maintenance and experimental preparation .... 21

2.1.1. *Aplysia* maintenance .... 21

2.1.2. Light and dark cycle .... 22

2.2. Preparation of *Aplysia* for the experiment .... 22

2.2.1. Anaesthesia .... 23

2.2.2. Parapodectomy .... 23

2.2.3. Implantation of electrodes .... 25

2.3. Equipment for training and testing ........ 28
2.3.1. Stimulus applications ........ 29
2.4. Experimental design ........ 33
2.5. Experimental proceeding ........ 34
2.5.1. Pre-test and classical conditioning ........ 34
2.5.2. 24h-test and extinction training ........ 35
2.5.3. 48h- and 72h-test ........ 36
2.5.4. Acoustic guiding of the stimulations ........ 36
2.6. Sleep deprivation ........ 37
2.7. Behavioural analysis ........ 38
2.7.1. Scoring of withdrawal durations ........ 38
2.7.2. Monitoring of sleep and wake activity ........ 39
2.8. Statistical analysis ........ 40
2.8.1. A priori selection of animals excluded from statistical analysis ........ 41

**3. Results ........ 42**

3.1. Learning outcome ........ 42
3.1.1. Classical conditioning ........ 42
3.1.2. Extinction training ........ 46
3.1.3. Trial means in conditioning and tests ........ 46
3.1.4. 72h follow-up test ........ 51
3.2. Sleep quanta of experimental groups ........ 52
3.2.1. Sleep quanta in 6 and 24 hours after training ........ 53
3.2.2. Sleep quanta in 48 hours after training ........ 54
3.3. Correlation of sleep quanta and learning ........ 56
3.3.1. Sleep during 6 h after training and siphon withdrawal durations ........ 56
3.3.2. Sleep in 24 hours and siphon withdrawal durations ........ 58
3.4. Supplemental Results ........ 60
3.4.1. Preliminary experiments ........ 60
3.4.2. Supplemental analysis of the main experiment ........ 66
3.4.3. Physical condition of experimental animals ........ 73

**4. Discussion** ..... **75**

4.1. Classical conditioning and sleep ..... 75

4.1.1. Conditioning was effective ..... 75

4.1.2. Classical conditioning was not impaired by sleep deprivation ..... 76

4.1.3. Sleep levelled learning ..... 78

4.2. Extinction ..... 79

4.3. Study design ..... 85

4.3.1. Low unconditioned stimulus intensity ..... 85

4.3.2. Duration of sleep deprivation and memory effect ..... 86

4.3.3. Homoeostatic impact and stress induction of sleep deprivation method ..... 87

4.4. Study strengths ..... 89

4.5. Conclusion ..... 91

**5. Summary** ..... **93**

**5. Zusammenfassung** ..... **95**

**6. References** ..... **97**

**7. Eigenanteilserklärung** ..... **109**

**8. Veröffentlichungen** ..... **110**

**9. Danksagung** ..... **111**

# II. List of figures and tables

## Figures

Figure 1.1: *Aplysia californica* anatomy. .... 10
Figure 1.2: Standard procedure of classical conditioning. .... 13
Figure 1.3 Protocol design for extinction of classical conditioning. .... 14
Figure 2.1: Maintenance of *Aplysia californica*. .... 21
Figure 2.2: Anaesthesia of *Aplysia* in ice water. .... 23
Figure 2.3: Parapodectomy. .... 24
Figure 2.4: *Aplysia californica* after parapodectomy. .... 25
Figure 2.5: Electrode implantation in *Aplysia*. .... 27
Figure 2.6: Tail of an *Aplysia californica* with one implanted electrode. .... 28
Figure 2.7: Experimental workspace layout for classical conditioning. .... 29
Figure 2.8: Triggering of the siphon withdrawal reflex. .... 30
Figure 2.9: Apparatus used for application of electric shocks and circuit diagram. .... 32
Figure 2.10: Experimental design. .... 33
Figure 2.11: Stimulus applications in the pre-test and conditioning. .... 35
Figure 2.12: Transcript of the guiding audio for tests and trainings. .... 36
Figure 2.13: Video recording setup .... 39
Figure 3.1: Siphon withdrawal duration before and 24 hours after classical conditioning. .... 43
Figure 3.2: Prolongation of withdrawal durations in acquisition, 24h- and 48h-test. .... 45
Figure 3.3: [A] – [D]: Individual siphon withdrawal durations in trials of all tests and acquisition of all animals. .... 51
Figure 3.4: Siphon withdrawal duration as difference to pre-test of *Aplysia* before and 24 hours after classical conditioning and 24 and 48 hours after extinction training. .... 52
Figure 3.5: Sleep duration 6 and 24 hours after conditioning training in sleep deprived and control group animals. .... 54

Figure 3.6: Sleep duration in 48 hours after conditioning training, in sleep deprived and control group animals. .......................................... 55
Figure 3.7: Correlation of sleep duration during 6 hours after training and behavioural outcome. ................................................................ 57
Figure 3.8: Correlation of sleep duration in 6 hours after training and behavioural outcome. ................................................................ 59

## Tables

Table 3.1: Behavioural outcome 24 hours after classical conditioning of *Aplysia californica* trained with either 4.5 V or 10 V. ................... 60
Table 3.2: Resulting current for different voltages applied to *Aplysia californica* via implanted electrodes. .......................................... 61

## Supplemental Figures

Supplemental figure 1: Learning effect of classical conditioning of the siphon withdrawal reflex with 4.5 and 10 V shocks. ....................................................................... 62
Supplemental figure 2: Behavioural outcome of classical conditioning of the siphon withdrawal reflex with a tail shock of 10 V / 3.9 mA in *Aplysia californica*. ............................. 64
Supplemental figure 3: Sleep duration of 9 animals during night with food odour and control night. .......................................... 65
Supplemental figure 4: Siphon withdrawal durations at three testing points. ...................................................................... 66
Supplemental figure 5: Siphon withdrawal durations at three testing points and conditioning. .......................................... 67
Supplemental figure 6: Siphon withdrawal durations ordered by trial in pre-, 24h- and 48h-test ([A], [B] and [C] respectively) in *Aplysia*. .......................................... 70

Supplemental figure 7: Siphon withdrawal duration before and 24 hours after classical conditioning and 24 and 48 hours after extinction training. ........................................... 71
Supplemental figure 8: Diameters of the electrodes for shock application and resulting current................................................ 72
Supplemental figure 9: Body weight of *Aplysia* in classical conditioning experiment. ............................................................. 73
Supplemental figure 10: Weight loss of SLEEP and WAKE group animals in the experiment..................................................... 74

# III. List of abbreviations

| | |
|---|---|
| 2D | two-dimensions |
| AC | alternating current |
| A / mA | ampere / milliampere |
| ANOVA | analysis of variance |
| *Aplysia* | *Aplysia californica* |
| CS | conditioned stimulus |
| CR | conditioned response |
| *Drosophila* | *Drosophila melanogaster* |
| EEG | electroencephalogram |
| e.g. | exempli gratia, Latin for “for example” |
| EMG | electromyogram |
| EOG | electrooculogram |
| FMRFamide | amide made of Phe-Met-Arg-Phe |
| GABA | gamma-amino butyric-acid |
| h | hours |
| i.e. | id est, Latin for "that is" |
| LED | light-emitting diode |
| LFI | learning that food is inedible |
| lx | lux (unit of illuminance) |
| min | minute |
| n | number of subjects |
| NMDA | N-methyl-D-aspartate |
| REM | rapid eye-movement |
| s | second |
| SWS | slow-wave sleep |
| SD | sleep deprivation |
| SD | standard deviation (in results section) |
| SEM | standard error of the mean |
| US | unconditioned stimulus |
| UR | unconditioned response |
| UV | ultra-violet |
| V | voltage |
| Ω / kΩ | ohm / kiloohm |

# 1. Introduction

## 1.1. Learning and memory

Learning and memory are essential for an animal to adapt to its environment. If an organism can learn about the features of dangerous situations, it can avoid such situations in the future. Learning is therefore usually expressed in a change of behaviour. The neurophysiological correlates of learning and memory are well summarised by Majumdar et al. (2012): "*Learning changes the efficacy and number of synaptic connections. Memory is the maintenance of that altered state over time.*"

In a basic learning example to illustrate this definition, two sensory stimuli are presented to an organism with short temporal distance. Each stimulus will excite sensory neurones that will transduce their signal via synapses to further neurones. At some point, the projections of both pathways converge to a mutual output and here, the synapse will be strengthened. This principle was first described by Hebb (1949). After repeated paired stimulus appearances, the cellular changes will be stabilised to from memory. Learning and memory exist in different levels of complexity, both shall be further elucidated in the following.

### 1.1.1. Learning

Basic types of learning have been described by W. Thorpe (1951). He suggested six learning patterns, namely habituation, classical conditioning, instrumental conditioning, imprinting, latent learning and observational learning.

Habituation is the gradual decline of an animal's reaction to a recurrently presented stimulus.

Conditioning is divided in classical and operant conditioning. In classical conditioning, an insignificant stimulus (conditioned stimulus, CS) is paired with another, strong stimulus shortly thereafter (unconditioned stimulus, US). The unconditioned stimulus will always provoke a specific reaction in the organism. Through training, the animal will learn that the conditioned and unconditioned stimulus are connected. Therefore, the reaction to the insignificant stimulus will

resemble the specific reaction to the strong stimulus (Pavlov 1927). For instrumental, or operant conditioning, the subject learns the connection between a task it has to complete and a thereof resulting reward or avoidance of punishment (Skinner 1938).

Imprinting refers to learning about the characteristics of a stimulus or object that will later be preferred to others or treated in a specific way. Imprinting only happens within a critical time period and is characterized by irreversibility and long retention. A well-known example is learning their parents' identities in birds (Lorenz (1937); Bolhuis and Honey (1998)).

Latent learning is acquired without special attention of the organism to the learned content and does not require reinforcement (Thistlethwaite 1951).

During observational learning, a subject observes the behaviour of another individual and is able to understand and reproduce this behaviour (Bandura and Huston (1961); Fryling, Johnston and Hayes (2011)).

Beyond these basic categories, an acquired memory can be modified by further learning. One example with relevance to this work is extinction learning, which is thought to serve an adaptive function in a changing environment. Extinction can happen in classical or operant conditioning (Manahan-Vaughan, Wolf and Güntürkün 2016). Through extinction learning, an animal learns, that the association of two stimuli or the causality between behaviour and subsequent stimulus is no longer valid. Most commonly, in the extinction training, the indicating stimulus or the specific behaviour is not reinforced. Extinction is different to forgetting in that it requires additional training and that the primarily acquired memory will spontaneously recover (Myers and Davis 2002, Rescorla 2004).

### 1.1.2. Memory

Memory is commonly divided into three stages: encoding, consolidation and retrieval. Encoding refers to processing of an input, in order to form a neuronal representation, i.e. a memory trace. During consolidation, this trace is stabilized and integrated into existing knowledge. For some types of learning, consolidation

is sleep-dependent. In these cases, consolidation can be impaired by sleep deprivation. The recall of stored memory is referred to as retrieval.

The consolidating process can be disrupted by chemical agents such as anisomycin (a protein synthesis inhibitor) and NMDA-antagonists like ketamine or phencyclidine. In case of sleep-dependent consolidation, it is possible to enhance the consolidation process. For this purpose, a context clue, which was present during acquisition is offered when memory is consolidated during sleep (Rasch et al. 2007).

Memory can further be classified depending on its temporal persistence. Memory lasting for a time span of approximately 30 minutes is commonly referred to as short-term memory. Long-term memory usually lasts for more than 24 hours and requires protein synthesis and degradation (Jarome and Helmstetter 2014).

Memory as the stored content of previous learning, was historically divided into non-declarative (implicit) and declarative (explicit) memory (Squire and Zola 1996). A memory is non-declarative or procedural if it implies an action or its content is subconsciously stored information. Declarative memory comprises knowledge about facts and events and includes a spatio-temporal dimension. Further, memory can be distinguished into associative or non-associative. Associative memory is the knowledge about the connection of two stimuli. The result of classical conditioning is associative memory.

#### 1.1.2.1. Memory in invertebrates

Invertebrates, although being rather simple organisms, exhibit a broad range of learning and memory. *Aplysia*, as many other invertebrates is capable of non-associative and associative learning (Hawkins and Byrne 2015, Byrne and Hawkins 2015). More complex memory forms like spatial memory can be found in bees (Dyer, Berry and Richard 1993) and *Drosophila* (Foucaud, Burns and Mery 2010). Octopus can even perform complex learning paradigms, including spatial and observational learning (Zarrella et al. 2015).

## 1.2. Sleep

Although, sleep appears to be a non-active state, within the body, vital processes are conducted autonomously. A total lack of sleep will finally result in death, as demonstrated in rats by (Rechtschaffen et al. 1983). Sleep is a state opposed to wakefulness, a resting state, which can be found in all animals that have been investigated so far (Campbell and Tobler (1984); Cirelli and Tononi (2008)). Sleep durations vary greatly at different ages and throughout phylogeny.

To identify sleep in any species, there are four essential features of sleep on the behavioural level (Vorster and Born 2015):

- delayed responses to external stimuli
- increased arousal threshold
- fast reversibility unlike in coma or hibernation.
- rebound sleep after sleep deprivation

Other typical but not essential observations are a specific body posture and twitches during sleep. Sleep can further be induced by hypnotics and postponed by stimulants. During development sleep is often increased. Typically, animals exhibit pre-sleep rituals or sleep in dedicated places.

### 1.2.1. Physiology of sleep

Behavioural observation is the most universal way to assess sleep. It can be supported by physiological monitoring of which an essential component is the measuring of brain activity. In *Drosophila*, Nitz et al. (2002) reported a general reduction of brain activity during sleep by means of local field potential recording. In mammals and birds, sleep can be assessed using electroencephalography (EEG). Together with the simultaneous recording of electromyogram (EMG) and electrooculogram (EOG), specific sleep states can be differentiated: rapid eye-movement sleep (REM-sleep) and slow wave sleep (SWS). In REM sleep or paradoxical sleep, characteristic rapid eye movements occur, the EEG signal resembles wakefulness. Most voluntary muscles are paralyzed, which can be seen in the EMG. Therefore, in mammals and birds, sleep is divided into REM

and non-REM sleep. SWS is a phase within Non-REM sleep, that is characterized by slow waves in the EEG (0.5 – 4.0 Hz, wakefulness ca. 13 - 40 Hz). Observational findings suggest the existence of different sleep phases even in invertebrates: Cassill et al. (2009) found rapid antennal movement in sleeping fire ants. Distinct sleep stages in invertebrates were described in cockroaches and bees (Tobler and Neuner-Jehle (1992); Zwaka et al. (2015)). Local field potential recordings in a lizard species revealed that even reptiles might exhibit slow wave sleep and REM sleep (Shein-Idelson et al. 2016).

During sleep, other body functions change, like the heart rate and blood pressure. These are generally reduced while sleeping but can fluctuate significantly during REM sleep. Further, during REM sleep, the body loses its ability to control temperature (Parmeggiani 2003).

#### 1.2.1.1. Sleep regulation

Many aspects of sleep's regulation are similar or equal in different species suggesting an evolutionary conservation. The occurrence of sleep is mostly circadian, following the inner clock which is ubiquitary provided by the hormone melatonin (Pevet, Klosen and Felder-Schmittbuhl 2017). The amount of slow wave sleep increases after previous sleep deprivation, indicating that it is homoeostatically regulated (1995 Tobler). Hormones which are involved in the regulation of sleep are highly comparable for vertebrates and invertebrates (Zimmerman et al. 2008). Gamma-amino butyric-acid (GABA) inhibits wakefulness-promoting areas in mammals (Gottesmann 2002), zebrafish (Nishimura et al. 2015) and invertebrates as fruit flies (Agosto et al. 2008). In the worm C. elegans, FMRFamide-like peptide 11 (flp-11) is the analogue of GABA (Turek et al. 2016). The salt-induced protein kinase SIK3 is important for the regulation of sleep in humans as well as mice (Funato et al. (2016), Honda et al. (2018)). Sleep durations are increased by a gain-of-function mutation of SIK3 orthologues in fruit flies, zebrafish and C. elegans (Funato et al. (2016), Levitas-Djerbi and Appelbaum (2017)).

On the other side, wakefulness is supported by catecholamines and other biogenic amines like histamine in vertebrates (Monti and Jantos (2008), Saper,

Scammell and Lu (2005)). Likewise in invertebrates, the orthologue of noradrenaline named octopamine promotes wakefulness as shown in *Drosophila* (Crocker and Sehgal 2008). Serotonin in humans is involved in regulation of both sleep and wakefulness (Imeri and Opp (2009); Ursin (2002)) and was found to induce sleep in fruit flies (Yuan, Joiner and Sehgal 2006).

#### 1.2.1.2. Sleep adapts

Whether sleep occurs diurnally or nocturnally, is determined by the ecological niche. Even in the same genus this may be different, as in *Aplysia californica* (diurnal activity) and *Aplysia fasciata* (nocturnally active) (Lyons et al. 2005). Sleep is not necessarily a global brain function, it can occur in parts of the brain only, so called local sleep. This phenomenon is found as an adaptation to special environmental requirements: dolphins exhibit unihemispheric sleep, as they would be under the risk of drowning if they stopped swimming (Mukhametov 1987, Sekiguchi, Arai and Kohshima 2006). Another example are migrating birds, that need to sleep at least with half of the brain during long flights (Rattenborg et al. 2016). The observed local sleep is SWS, as REM sleep is incompatible with ongoing motion, because skeletal muscles are paralysed. But also non-migrating birds and some reptiles exhibit unihemispheric sleep, due to specific circumstances (Rattenborg, Amlaner and Lima 2000). Also in humans, slow wave sleep and sleep spindles were found to occur regionally (Nir et al. 2011, Siclari and Tononi 2017). Furthermore, local field potential recordings of sleep deprived, awake mice, showed regional SWS in small neuron populations (Vyazovskiy et al. 2011).

### 1.2.2. The function of sleep

While regulatory mechanisms are quite well understood for many different species, the function of sleep is not yet fully elucidated. Either external factors like the day- and night cycle, induced in living organisms a circadian phase of inactivity. Consequently, specific body processes were scheduled into the sleeping phase for efficient performance. Alternatively, there is a core function of sleep, that requires animals to rest in a determined way. The fact that during sleep, individuals remain unconscious and paralysed and thus extremely

vulnerable, affirms that sleep must serve a vital purpose. Several functions are considered in search of this hypothetic pivotal sleep process:

1) Sleep for the immune system:

Sleep and especially SWS promotes the adaptive immune system (Westermann et al. 2015). For example, after a vaccination, subjects that slept during the subsequent night, had higher levels of antigen-specific T-helper cells, compared to sleep deprived participants (Lange et al. 2003, Prather et al. 2012). This proved to be a long-term effect, even measurable after one year (Lange et al. 2011). Sleep deprivation in turn results in higher levels of pro-inflammatory immune cytokines (Besedovsky, Lange and Born 2012).

2) Neuronal clearance during sleep

Xie et al. (2013) described, that during sleep in mice, interstitial space in the brain increased by 60%. This resulted in an accelerated flow and exchange of cerebrospinal fluid from artery to vein. The higher flow led to faster removal of ß-amyloid, accumulations of which are associated with Alzheimer’s disease (Cedernaes et al. 2017, Ju, Lucey and Holtzman 2014). Tau protein, also associated with Alzheimer’s and other neurodegenerative diseases, was increased in mice after being sleep deprived every day for 4 hours during 8 weeks (Di Meco, Joshi and Praticò 2014). Also, anesthetically induced sleep-like states were shown to support metabolic clearance (Xie et al. 2013). This might indicate that unconsciousness is necessary for this process.

3) Sleep to save energy

That sleep serves to economise energy, was suggested amongst others by Berger and Philips (1993) and Schmidt (2014). The energy consumption during sleep is reduced by at least 10%. The saving is even higher in cold environments and furthermore if only the brain energy consumption is taken into account (Berger and Phillips 1995). Energy saving during sleep stands against the risk of predation in this vulnerable state. As the amount of energy saved is rather small, it does not appear relevant enough to induce the development of such a dangerous body state that also consumes a great amount of time. Another interesting finding contradicts the energy saving hypothesis: hibernating and

torpid animals repetitively heat up their body, and sleep during this euthermic phases (Daan, Barnes and Strijkstra 1991). Hibernation and torpor are states dedicated to reduction of energy consumption; therefore sleep's function is unlikely just another way of conserving energy. To the contrary, in this context energy is even spent to heat up the body, in order to be able to sleep. This underlines that sleep most certainly serves a different and evidently vital process.

4) Sleep's memory function:

The link between memory and sleep has intensely been studied across species (see reviews Rasch and Born (2013), Vorster and Born (2015), Sara (2017)). Notably, not all types of memory are equally affected by sleep. For example, encoding and consolidation of hippocampus-dependent but not of hippocampus-independent memory seems to benefit from sleep in mammals (Inostroza and Born 2013). Of the three phases of memory, mainly consolidation was found to interact with sleep.

### 1.2.3. Sleep's role for memory consolidation

Many hypotheses intend to explain the way through which sleep interacts with memory consolidation. Most influential are the active system consolidation and the synaptic homeostasis theory.

#### 1.2.3.1. Active system consolidation theory

The active system consolidation theory is based on the two-stage model of memory. The idea behind this model is, that learned content is initially encoded into a temporary storage and later redistributed for long-term storing (Buzsaki 1989). In case of declarative memories, the hippocampus is the neuronal correlate of the transitory store. This type of storage is fast acting but unstable. Through multiple reactivations, memory content is transferred into long-term storage and integrated into earlier acquired memory (Born and Wilhelm 2012). Slow-wave sleep is thought to be the sleep state where such reactivations take place.

Cortical slow wave oscillations induce synchronised hippocampal sharp wave-ripple and thalamo-cortical spindle activity. Sharp wave-ripples are interpreted as

the neurophysiological correlate of hippocampal memory replay, spindles as reflections of memory storage reorganization (Rasch and Born 2013). Sleep is thus not a pure off-line phase that allows for undisturbed processing. Rather the "sleep-mode" of the brain is caused by memory reorganization processes and therefore represents an active function. Evidence for this theory comes from studies by Schreiner and Rasch (2015) and Rudoy et al. (2009). After a learning task, during slow wave sleep, subjects were presented clues of the acquisition context. The context clue triggered reactivation of the learned content and the consolidation process was enhanced. This was measurable in a subsequent memory test during wake by improved performance.

#### 1.2.3.2. Synaptic homeostasis theory

Suggested by Tononi and Cirelli (2003), this theory is based on the finding, that synaptic potentiation occurs during wakefulness. Synaptic potentiation happens due to an increase in number and strength of synapses (Bushey, Tononi and Cirelli 2011). If this growth was unregulated, it would lead to exceedingly high demand in energy and space. This would eventually result in a saturated nervous system, losing its capacity to learn, i.e. its plasticity. Tononi and Cirelli suggest, that the off-line state of the brain during sleep, provides the preconditions for undisturbed downscaling. Downscaling refers to a reduction of synaptic strength and amount to restore the system's homoeostasis. That this happens during sleep could be testified in rodent cortices (Liu et al. 2010). The way by which synaptic homoeostasis supports learning is rather indirect: downscaling includes all synapses, but those that have been potentiated by learning during wakefulness, will proportionally remain stronger. The signal-to-noise ratio is thereby increased, which in turn is thought to benefit performance (Huber et al. (2004); Tononi and Cirelli (2006)). Consolidation is therefore a secondary effect, resulting from synaptic down-scaling. This theory does not involve specific sleep phases and is therefore universally applicable to all species that exhibit enhancement of learning through sleep.

## 1.3. *Aplysia* as a model for sleep and memory

*Aplysia* is a sea slug with several subgenera, indigenous in different parts of the world, mostly in tropical and subtropical tide zones. *Aplysia californica* shows a stable circadian rhythm with diurnal activity (Vorster et al. 2014). The slug feeds off algae and can grow up to about 75 cm in length and 15.9 kg. Animals used in the laboratory typically don’t exceed 20 cm length and 400 g body weight. Pioneering work on the electrophysiological and cellular mechanisms of learning and memory, has been performed using *Aplysia* as a model organism. This is due to its ideal suitability for electrophysiology: the neural system of *Aplysia* only comprises about 20.000 neurons. These are organized in 5 paired ganglia, spread across the animal. Additionally, the neurons are quite large, the biggest reaching up to 1 mm in diameter (Moroz 2011). Therefore, it is possible to identify some individual neurons with the bare eye.

### 1.3.1. Anatomy of *Aplysia*

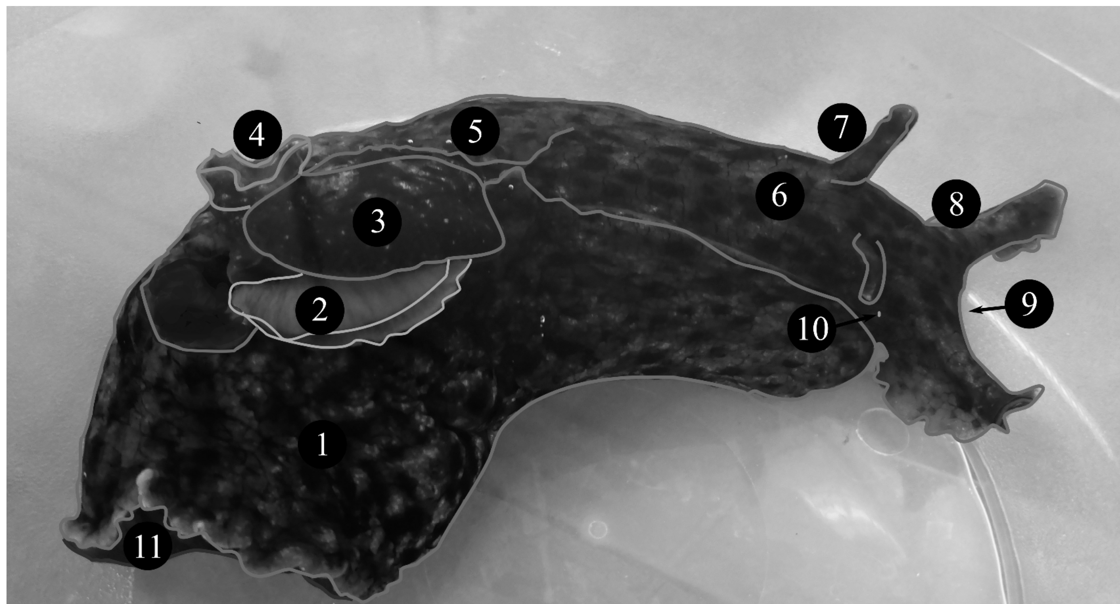



**Figure 1.1: *Aplysia californica* anatomy.**

❶ tail
❷ gill
❸ mantle
❹ siphon
❺ parapodium
❻ head
❼ rhinophore
❽ tentacle
❾ mouth
❿ eye
⓫ foot

The parapodia were clipped to reveal the structures on the back.

Figure 1.2 shows an *Aplysia* with its relevant anatomical structures. The head of *Aplysia* holds two olfactory organs: the tentacles ⑧ and the rhinophores ⑦. Rhinophores are rather for distance chemoreception, tentacles for contact chemo- and mechanoreception (Cummins et al. 2009). *Aplysia* possess eyes ⑩, but rhinophores and tentacles are additionally sensitive to light (Jacklet 1980) as well as the whole body wall, although to a fewer extend. The mouth ⑨ of *Aplysia* is located between the tentacles, on the ventral side of head. The radula inside the mouth can be used as a pincer to grasp food like macro algae.

*Aplysia*'s respiratory organ, the gill ②, lies on the dorsal side underneath a residual shell, which is enclosed in the mantle ③. The posterior skin of the mantle extends to form a cylindric funnel, called siphon ④. It serves an excretory purpose: it drains what comes from the anus, water from the gill, ink from the purple gland and opaline from the opaline gland (Byrne, Castellucci and Kandel 1974). The siphon is furthermore mechanosensitive: a tactile or light electric stimulus causes the defensive siphon- and gill withdrawal reflex. Here both siphon and gill are retracted for several seconds (Kupfermann, Carew and Kandel 1974). The location of the siphon is on the back of the snail, usually hidden from view by two pieces of skin called parapodia ⑤.

### 1.3.2. Chemical defense

If *Aplysia* is exposed to a strong, noxious stimulus, additionally to the siphon and gill withdrawal reflex, *Aplysia* will release ink and opaline. Ink is a liquid excretion of dark purple colour, which is unpalatable to most predators like Navanax and anemones (Leonard and Lukowiak 1986). It contains toxins and pigment of the animal's diet (Nolen et al. 1995). Opaline is transparent or white, highly viscous and contains free amino acids and other constituents, that stimulate chemo sensors of predators (Nusnbaum and Derby 2010).

When in vital danger, *Aplysia* releases the two substances to defend itself. It is hypothesised, that ink acts as a decoy and opaline mimics food intake, this combination is called "phagomimicry". Opaline is complementing the deterrent effect of ink: the high concentration of chemical stimulators within the sticky

secretion causes a high-magnitude, long-lasting stimulation. This leads to sensory disruption in the predator (Kicklighter et al. (2005), Derby et al. (2007), Sheybani et al. (2009)) and consequently, *Aplysia* will be released (Nolen et al. 1995).

### 1.3.3. Learning paradigms in *Aplysia*

In *Aplysia*, sensitisation is the most extensively studied learning paradigm, both on the behavioural and molecular level (Pinsker et al. 1973, Greenberg et al. 1987, Scholz and Byrne 1987, Walters 1987, Knapp 1988, Cleary et al. 1991, Cleary, Lee and Byrne 1998). Protocols are also established for habituation, classical and operant conditioning (Carew, Castellucci and Kandel 1971, Carew, Pinsker and Kandel 1972, Marcus et al. 1988, Cohen et al. 1997, Walters, Carew and Kandel 1979, Carew, Walters and Kandel 1981b, Carew, Hawkins and Kandel 1983, Hawkins 1984, Hawkins et al. 1989, Colwill, Goodrum and Martin 1997, Cook and Carew 1986, Hawkins, Clark and Kandel 2006, Krishnan et al. 2016a, Vorster and Born 2017). Beyond that, secondary modifications of acquired memory have been performed, like dishabituation and reversal training like extinction (Marcus et al. 1988, Rankin and Carew 1988, Vorster and Born 2018). Paradigms relevant to this experimental work include classical conditioning and extinction of the siphon withdrawal reflex as well as its sensitization.

#### 1.3.3.1. Classical conditioning and extinction of the siphon withdrawal reflex

The main experiment of this work is based on an experiment by Carew et al. (1981b). The group showed, that the prolonged duration of the siphon withdrawal reflex of *Aplysia*, can be acquired through conditioning and the newly learned response can afterwards be extinguished.

In classical conditioning, two stimuli are sequentially applied to an experimental subject: primarily the conditioned stimulus (CS), a neutral stimulus, which causes a minor reaction in the animal. This stimulus serves as a cue for the subsequent presentation of a stronger stimulus, the unconditioned stimulus (US). The US will

in any circumstance provoke a reflexive behaviour, the unconditioned response (UR). By repeated paired exposure to the CS and US, the animal will learn to associate the two events. In a memory test after conditioning, those animals that learned, will exhibit the reflexive behaviour when solely the weak, conditioned stimulus is applied. The reflexive response (UR) is now called conditioned response (CR), as it is associated to the conditioned stimulus (CS). Figure 1.3 provides a graphical overview of the protocol design for classical conditioning:

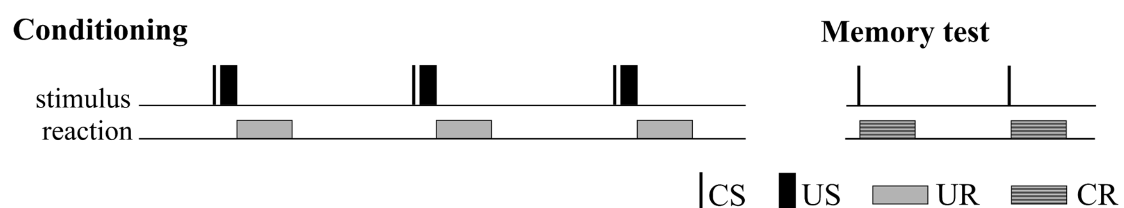



**Figure 1.2: Standard procedure of classical conditioning.**
CS = conditioned stimulus, US = unconditioned stimulus, UR = unconditioned response, CR = conditioned response.
The unconditioned stimulus is always followed by the unconditioned response. After repeated paired presentation of the CS and US the CS will be sufficient to elicit the UR, which is then called CR.

In conditioning of the siphon withdrawal reflex of *Aplysia*, a light touch of the siphon, for example with a chop stick, serves as the conditioned stimulus (CS). It causes a short withdrawal of the siphon of several seconds. A strong stimulus to another part of the body, like an electric shock (US), will also trigger siphon- and gill withdrawal. Yet in this case, the duration of the reflexive withdrawal is longer, i.e. more than one minute (UR). *Aplysia* can learn to show prolonged siphon withdrawal in response to the light siphon touch. To this aim, the electric shock is applied directly after a weak siphon touch in several trials. In a retrieval test after the conditioning trials, the response to siphon touch will resemble the reaction to the electric shock (CR).

A learned association can be reversed, when the conditioned stimulus is presented several times without reinforcement of the unconditioned stimulus. In

a subsequent memory test, the conditioned stimulus will no longer provoke the conditioned response (figure 1.4).

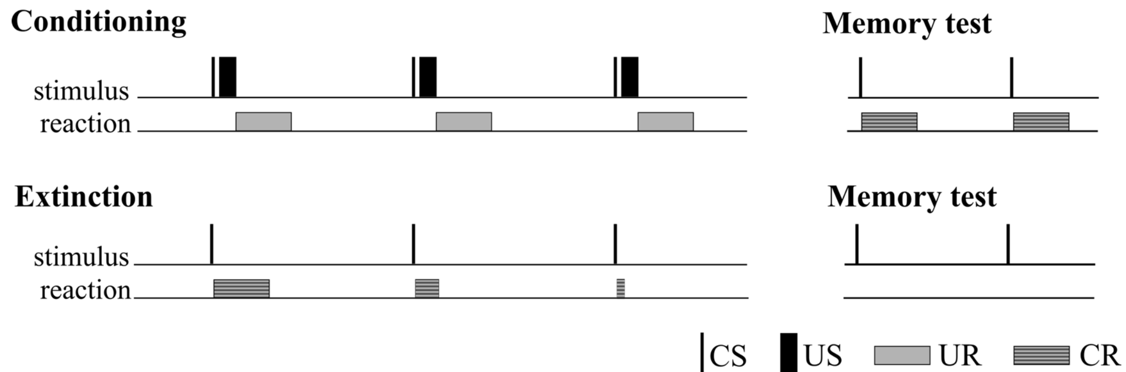



**Figure 1.3 Protocol design for extinction of classical conditioning.**
CS = conditioned stimulus, US = unconditioned stimulus, UR = unconditioned response, CR = conditioned response.
When the conditioned stimulus is repeatedly presented without the subsequent unconditioned stimulus, the subject will cease to show the conditioned response.

Likewise, Carew et al. (1981b) found that the prolongation of the siphon withdrawal reflex can be extinct, if the CS is repetitively presented solely. The reaction to the weak stimulus thereafter will return to the initial short withdrawal. Extinction in *Aplysia* was also shown in an operant conditioning protocol (Vorster and Born 2018).

The classical conditioning used here is called **alpha-conditioning**. The characteristic feature is, that alpha-conditioning will enhance a pre-existing reflex, rather than associate a new reflex to the conditioned stimulus (Hawkins et al. 1989). The siphon withdrawal is elicited both by the tactile stimulus (CS) and the electric shock (US), but what differs are the durations. In order to measure the success of conditioning training, the siphon withdrawal durations before and after training are compared. If after training, tactile siphon stimulation makes the snail withdraw for much more than a minute, it learned to associate siphon touch to the electric shock.

In contrast, in **beta-conditioning** the unconditioned response is a behaviour that does not resemble the original reaction to the conditioned stimulus. Maybe the most common example for beta-conditioning is the Pavlovian experiment with

dogs, trained to associate the tone of a bell to feeding. Feeding as the unconditioned stimulus provokes the unconditioned response, which is salivation. The bell as the conditioned stimulus probably originally causes orientation towards the location of the bell. After associative training, the dogs salivated (unconditioned response) after hearing the tone of the bell in the expectance of food (Pavlov 1927).

Therefore, alpha-conditioning bears a relevant disadvantage when compared to beta-conditioning: without sufficient control groups, it cannot be differentiated from sensitization. Sensitized *Aplysia* also show prolonged siphon withdrawal. In fact, conditioning in *Aplysia* always holds a sensitizing effect (Hawkins et al. 1989, Kandel and Schwartz 1982).

#### 1.3.3.2. Sensitization of the siphon withdrawal reflex in *Aplysia*

For sensitization, *Aplysia* is repeatedly treated with a noxious stimulus. Earlier experiments used electric shocks, which were applied to different parts of the body like the head or tail region, following varying protocols (Pinsker et al. 1973, Castellucci and Kandel 1976, Fernandez et al. 2003, Cai et al. 2011). To measure sensitization, the siphon withdrawal reflex is triggered for example by tactile stimulation before and after training. In naïve animals, this will result in short siphon withdrawal, as described above. After training, a sensitized *Aplysia* will show significantly prolonged siphon withdrawal durations.

### 1.3.4. Sleep deprivation in *Aplysia*

#### 1.3.4.1. Methods of sleep deprivation

Methods used for sleep deprivation are not yet classified or standardised. Following literature research, three mechanisms can be distinguished: motivational, physical and chemical sleep deprivation.

Motivational sleep deprivation can be found in humans who simply by will (and maybe in expectancy of a reward) stay awake. In animals, this can be achieved for example by enriched and changing environment. Thereby, the animal is motivated to stay awake to be able to explore its surroundings.

An example for sleep deprivation through physical treatment, is shaking of bees (Hussaini et al. 2009) and *Drosophila* (Le Glou et al. 2012). Further methods are forced movement (Borbély and Neuhaus 1979) and gentle handling methods (Graves et al. 2003, Funato et al. 2016). Other examples are platform methods (Machado et al. 2004, Ravassard et al. 2009), where mice or rats are placed on a platform surrounded by water. When they fall asleep and loose muscle tonus, they fall into the water and are thereby woken up. This method rather induces REM-sleep deprivation, as loss of muscle tonus is typical for this sleep phase.

Chemical sleep deprivation refers to the application of a pharmacologic stimulant, preventing the subject from falling asleep like caffeine, dopamine, amphetamine or modafinil (Cai et al. 2009, Killgore et al. 2009, Wesensten, Killgore and Balkin 2005, Kume et al. 2005).

#### 1.3.4.2. Methods used in *Aplysia*

Both motivational and physical methods were used for sleep deprivation in *Aplysia*, chemical agents so far have not been administered. The first reported method to sleep deprive *Aplysia*, was context change every 30 min. This motivational approach was conducted by Vorster et al. (2014) combined with handling every 3 – 6 min. The method was adopted by Krishnan et al. (2016a) and Krishnan, Noakes and Lyons (2016b). Recently, Vorster and Born (2017) and Vorster and Born (2018) used a combination forced motion and gentle handling: when showing signs of sleep for more than one minute, the animals were removed from the surface they were attached to. This will wake up the animal because it triggers an action pattern called righting: when *Aplysia*'s foot is detached from a substrate, it will rotate its head until the foot touches a surface (Leonard and Lukowiak 1986). Removal was completed by means of a thin flexible plastic ruler, which was slid underneath the foot usually from head to the tail and thereby unsticking the snail.

#### 1.3.4.3. Alternatives to sleep deprivation

Several options exist to design an experiment, which investigates the influence of sleep on consolidation. For example, one can create two groups by making

use of the naturally occurring sleep and wakefulness. The sleep group would have to be trained before the resting phase. The control group, accordingly, at the beginning of the natural waking phase. This has been successfully performed in *Aplysia* by Vorster and Born (2017), Vorster and Born (2018). The disadvantage of this approach is the different training time in the two groups. Here, circadian processes might introduce confounding factors that make the groups less comparable.

Another possibility is to train both groups at the same time of the day and thereafter intentionally induce either sleep or wakefulness, at a time it usually does not occur. The internal problem of sleep deprivation is, that it is an intervention itself and not the pure opposite of sleep. The mere handling of the experimental animal might interfere with the memory processes, as shown by Cai et al. (2009).

A further consequence of sleep deprivation could be homoeostatic disbalances. These in turn might impair performance in the retrieval test, which could be misinterpreted as diminished memory. To conclude, any possible design will bear confounding factors arising from either circadian rhythms or manipulation. In this study, it was intended to minimize the potential of the sleep deprivation method to impair memory consolidation and retrieval by a short duration of sleep deprivation and reduction of necessary manipulation through motivation.

## 1.4. Previous work on conditioning memory and sleep in *Aplysia*

Sleep research only recently started to deploy invertebrate model organisms, which explains why the amount of such studies is rather limited. So far, four studies exist, which investigate the role of sleep in encoding, consolidation and extinction in operant conditioning in *Aplysia.* In the so-called learning that food is inedible (LFI) paradigm, *Aplysia* is presented with food wrapped inside a net. The animal learns that food can’t be swallowed and when testing 24 hours later, exhibits fewer biting trials, allowing the food to stay less time inside its mouth. In a study by Krishnan et al. (2016b) using the LFI protocol, formation of short- and

long-term memory was impaired by two nights of 6-hour sleep deprivation before training. They further investigated, if one night of recovery sleep could reverse the impairment of memory formation. One undisturbed night of rest only restored capability of memory formation in long-term but not short-term memory. Krishnan et al. (2016a) performed another LFI experiment and found that pre acquisition sleep deprivation of 9 hours, totally blocked the induction of both short- and long-term memory. The same amount of sleep deprivation strongly impaired consolidation of long-term memory.

This finding was confirmed by Vorster and Born (2017): two groups of *Aplysia* were trained at 8:00 am (Wake group) or 6:00 pm (Sleep group) with the LFI paradigm. The subsequent 17 hours were filled by wakefulness or sleep ad libitum, memory retention was tested thereafter. Both groups had learned that food inside the nets could not be swallowed, as reflected in fewer biting and reduced time the mouths touched food nets. But there was a clear beneficial effect of 17 hours of undisturbed sleep. These experiments combined, identify inhibitory operant conditioning in *Aplysia* as a sleep-dependent learning process.

Very surprisingly, extinction of LFI memory profited from 17 hours of wakefulness (Vorster and Born 2018). The authors suggest that by reactivation during the extinction training, the LFI memory was made vulnerable. Wakefulness thus supported forgetting the learned inhibition of food swallowing and *Aplysia* returned to the instinctive feeding behaviour.

## 1.5. Summary and aim of this work

This study was based on a publication by Hussaini et al. (2009), who showed that sleep had no effect in consolidation of classical conditioning in honeybees. However, extinction of classical conditioning was diminished after sleep deprivation. This is an example of sleep's differential effect on the various types of learning (Vorster and Born 2015). In this work it was now asked, whether classical conditioning is also consolidated independently of sleep in *Aplysia californica*, see also Thiede, Born and Vorster (2021). Thereto, two groups of animals were trained with one being sleep deprived for 6 hours after classical condition. By comparing the results of the sleep deprivation and the control group in a retrieval test performed 24 hours after conditioning, a potential impact of sleep deprivation could be detected.

Provided that this independency is evolutionary conserved, further conclusions on the function of sleep in memory formation could be drawn from this finding. If basic forms of learning like sensitization and conditioning are repeatedly found to be independent of sleep, one can postulate, that sleep is relevant for replay and redistribution of memory traces but not for monosynaptic processes.

That general principles in the function of sleep can be transferred even between far distant species, was shown by Zwaka et al. (2015). Based on a study in humans, they investigated whether sleep's function for memory consolidation would be similar in honeybees. Rasch et al. (2007) had shown that if a context odour was presented during learning and re-presented during slow-wave sleep, memory of 2D object-locations was enhanced. Zwaka et al. classically conditioned honeybees to exhibit the proboscis extension reflex to a heat stimulus. Training happened in the presence of a context odour, to which the interventional group was re-exposed during subsequent sleep. Bees that were presented with the odour, showed better memory in a retrieval test 24 hours after training. Odour re-exposure was even capable of enhancing memory that was acquired in a single-trial acquisition to reach the same level as standard 5-trial training.

Such astonishing direct parallels throughout phylogeny suggest a high evolutionary conservation of mechanisms which occur during sleep. To date, little

is known about the molecular effects of sleep on the level of the individual cell. To correlate sleep and associated cellular processes, the need for a simple neuronal network model arises. In these simple systems, underlying cellular events can be directly correlated to network functions. If learning can be found in simple circuits and this learning is additionally modified by sleep, cellular mechanisms of sleep could be unveiled.

This approach puts invertebrate models like *Aplysia* into focus. *Aplysia californica* is capable of different types of learning, reaching from simple forms like sensitization and classical conditioning to rather complex ones like inhibitory instrumental conditioning or extinction learning. At the same time, it has a simple neuronal system with ideal characteristics for electrophysiological and molecular analysis (Akhmedov et al. 2014). Therefore, *Aplysia* as a model organism might help to close the gap between sleep research on the behavioural and mechanistical level.

# 2. Material and Methods

A condensed version of the material and methods section was published in Thiede et al. (2021).

## 2.1. Animal maintenance and experimental preparation

### 2.1.1. *Aplysia* maintenance

The *Aplysia* were wild-caught in California (South Coast Bio-Marine, San Pedro, USA). Those used for the main experiment were collected in the month of March. Aiming at selecting animals of young age, only snails weighing between 80 – 125 g were chosen. For shipping, which lasted 5 days, each animal was kept inside an individual plastic bag, filled with sea water. In the laboratory, the bags were placed in two sea water aquaria. To allow for slow adaptation, the water inside the plastic bags was gradually exchanged for tank water. Thereafter, the *Aplysia* were placed inside individual floating plastic boxes of 10 cm x 15 cm x 30 cm, perforated on the sides to grant water circulation (figure 2.1).

**Figure 2.1: Maintenance of *Aplysia californica*.**
The picture was taken after feeding, each animal received a portion of ulva lactuca.

Sea water aquaria were of 340 l each, with in-system water circulation. Regularly, a quantity of water was removed and replaced with fresh artificial sea water (Instant Ocean, Aquarium Systems, Mentor, Ohio, USA). Water temperature of the aquaria was kept within a range of 15 – 16 °C, salinity between 35 g/kg – 36 g/kg. The tanks were equipped with a water preparation system, consisting of a live sand filling with coral gravel, a UV-lamp, a dribble filter with sponges, sintered glass rolls and active coal, as well as a skimmer to reduce residual proteins. At least every second day, the water purification system was cleaned. Feeding of animals before or after the experiment took place every second day, consisting of about 1 g of dried organic algae Ulva lactuca (ALGAplus Ltd, Portugal). The algae were macerated in sea water for approximately 15 – 30 min before being distributed. The experimental animals were last fed 5 days prior to conditioning and no food was provided during the experiment.

To monitor physical well-being, the weight of every *Aplysia* was measured upon arrival, before preparations for the experiment and directly before and after experiment. For weighing, animals were taken out of the water and placed directly onto the weighing scales.

### 2.1.2. Light and dark cycle

A time switch provided a biphasic light cycle, with light setting in at 07:00 and turning off at 19:00. During every experimental procedure, an additional LED bar lamp directly above the experimental area provided sufficient illumination for the cameras that recorded the animals' behaviour. During daytime, light intensity was > 100 lx and < 5 lx during the dark period.

## 2.2. Preparation of *Aplysia* for the experiment

First handling of the snails was performed on the fourth day post-arrival. There were two necessary procedures: Clipping of the parapodial skin and implantation of 2 electrodes. Clipping as the major intervention was performed at least 7 days, implantation at least 5 days before the experiment. All preparational and experimental procedures were completed in two animals simultaneously.

### 2.2.1. Anaesthesia

To anaesthetise the snails, the use of hypothermia proved to be most indulgent. This method had been used in *Aplysia* by Carew and Kandel (1977), Koch, Koester and Weiss (1984) and Fischer, Yuan and Carew (2000) and Lee et al. (2012). Canisters containing about 3 l of tank water were deep frozen. About 1 h before the anaesthesia, one canister was placed into the 16 °C water of the experimental table until half melted. The result was a thick mass of iced water at about - 2.5 °C, that was filled into a bowl. The snails were placed inside the mass to be totally covered.

The anaesthesia was considered sufficient, when the triggering of the siphon withdrawal reflex with a wooden chop stick did not provoke any response. In previous observations, most animals did not exhibit the reflex after 7 minutes in the iced water. The further steps were conducted outside the ice water. Figure 2.2 shows a bowl of iced water containing an *Aplysia*.

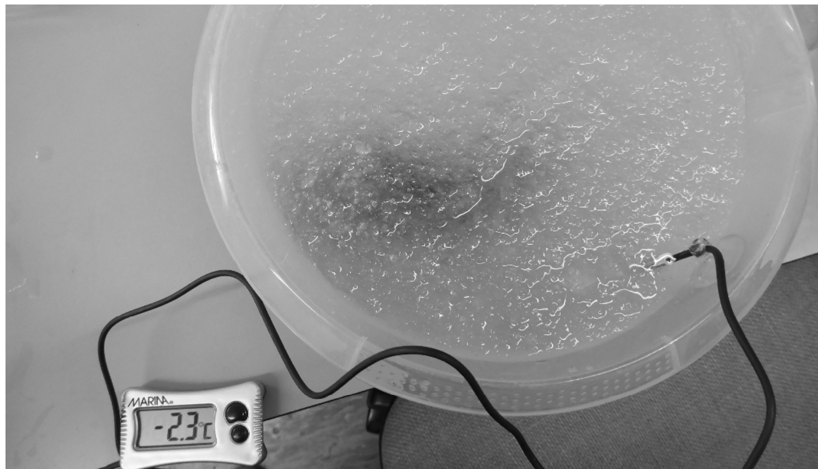


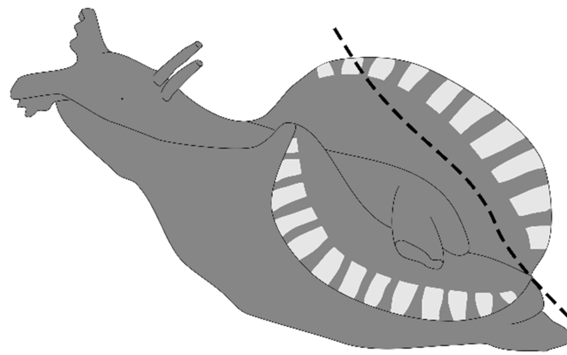

**Figure 2.2: Anaesthesia of *Aplysia* in ice water.**
The *Aplysia* were placed into the water for at least 7 minutes, until the siphon withdrawal reflex could no longer be elicited.

### 2.2.2. Parapodectomy

As in the original experiment by Carew et al. (1981b), the parapodia were clipped to attain visibility of the siphon in any relaxed or withdrawn state. This procedure was referred to as parapodectomy. The anaesthetised animal was laid on one side and the upper parapodial skin lifted with forceps. A haemostat was used to

compress the skin below the cutting edge. With surgical scissors, a stripe of parapodial tissue was cut off, beginning at the junction of parapodia and tail. This junction might easily be mistaken for the siphon (it is even called pseudosiphon), as both feature bright markings. Cutting as low as possible (Fischer et al. 2000) is thus important to avoid confusion. The upper part of the parapodia exhibit a white, shining marking, this was used as a guideline for cutting.

**[A]**



**[B]**

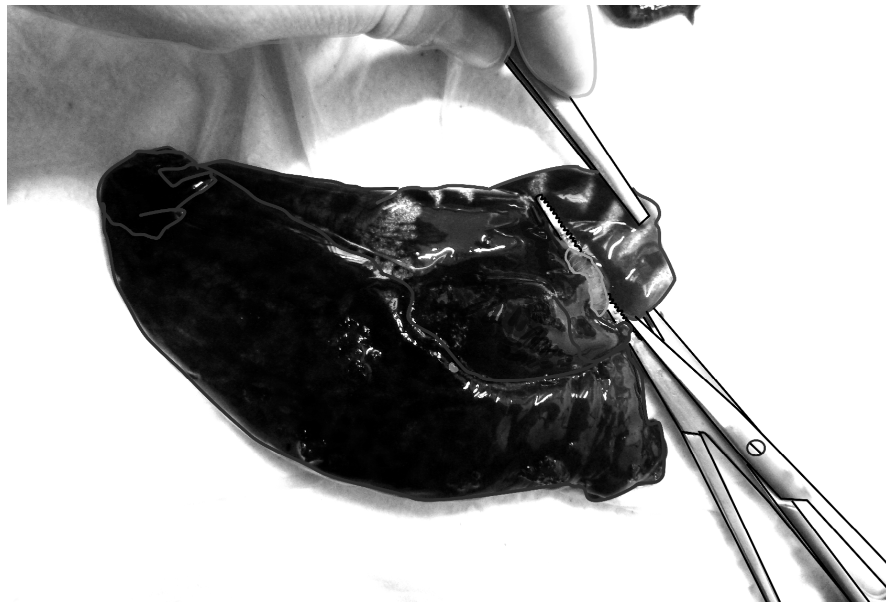


**Figure 2.3: Parapodectomy.**
[A] Along the dashed line, the parapodial skin was cut with surgical scissors. The inner side of the parapodial skin has a different skin pattern with bright shiny stripes. This was a guiding line for clipping.
[B] A haemostat compressed the skin below that line and forceps were used to lift up the parapodium.

After clipping, the muscles of the tissue contracted at the new margin and the wound sealed. This process was accelerated by the compression with the haemostat. Note that if the skin is cut within the fleshier part of the parapodia, contraction will be insufficient. In that case, a substantial amount of *Aplysia*'s haemolymph, will drain from the skin defect.

Subsequently the snail was turned to the other side and the procedure carried out equally on the other parapodium. The *Aplysia* were returned to the home tank where they rapidly regained normal body temperatures and started moving around.

**Figure 2.4: *Aplysia californica* after parapodectomy.**
The parapodial skin was partly removed to reveal the siphon. Once back in 15 °C sea water, the animals very quickly heated up after hypothermia and took up activity.

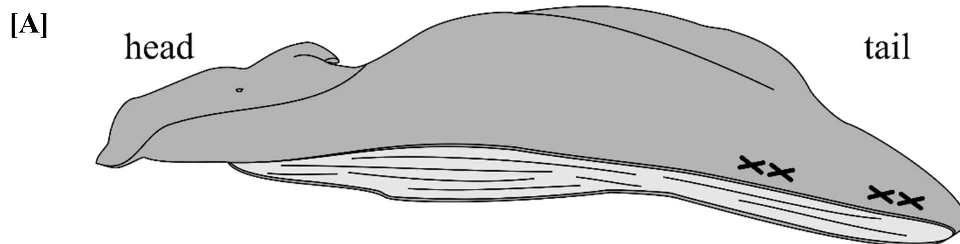
### 2.2.3. Implantation of electrodes

To provide equal current application in all animals, all snails were being implanted stainless steel wires (SS-5T/A, SS-8T/A, SS-10T/HH, Science Products GmbH, Germany) as electrodes. The product specifications represent three different thicknesses and flexibility: 125 µm, 200 µm and 150 µm respectively, A wires (annealed) are flexible, HH (half hard) rather stiff. Due to delivery retardation, it

was not possible to use the same wire in all animals. Yet the resulting current flow did not vary for different diameters. All wires were coated with Teflon, this provided isolation from sea water. Figure 2.5 depicts details of the implantation process.

Pieces of 15 cm of wire served as electrodes (see figure 2.5 [D]). 2 cm apart from one end of wire, 5 mm of the insulation was stripped to create a contact zone. The wire was implanted into the left tail region of the snail (figure 2.5 [C]), with a distance of 4 cm in the anaesthetised, relaxed animal (figure 2.5 [A]). When animals were back in the home tank and at normal body temperature, they contracted. Thereby the distance of the electrodes was reduced to approximately 1.5 cm. That the distances changed greatly depending on the muscle tonus of the *Aplysia*, was also reported by Carew et al. (1981b). The wire was inserted into the skin of the snail's tail by means of hypodermic (hollow) needles (figure 2.5 [B]). To secure the wire, it was bent in the contact area and both ends stuck out of the animal's skin. In that way, there was no contact of the bare wire with the sea water and current could only flow between the two electrodes inside the tissue.

[A]

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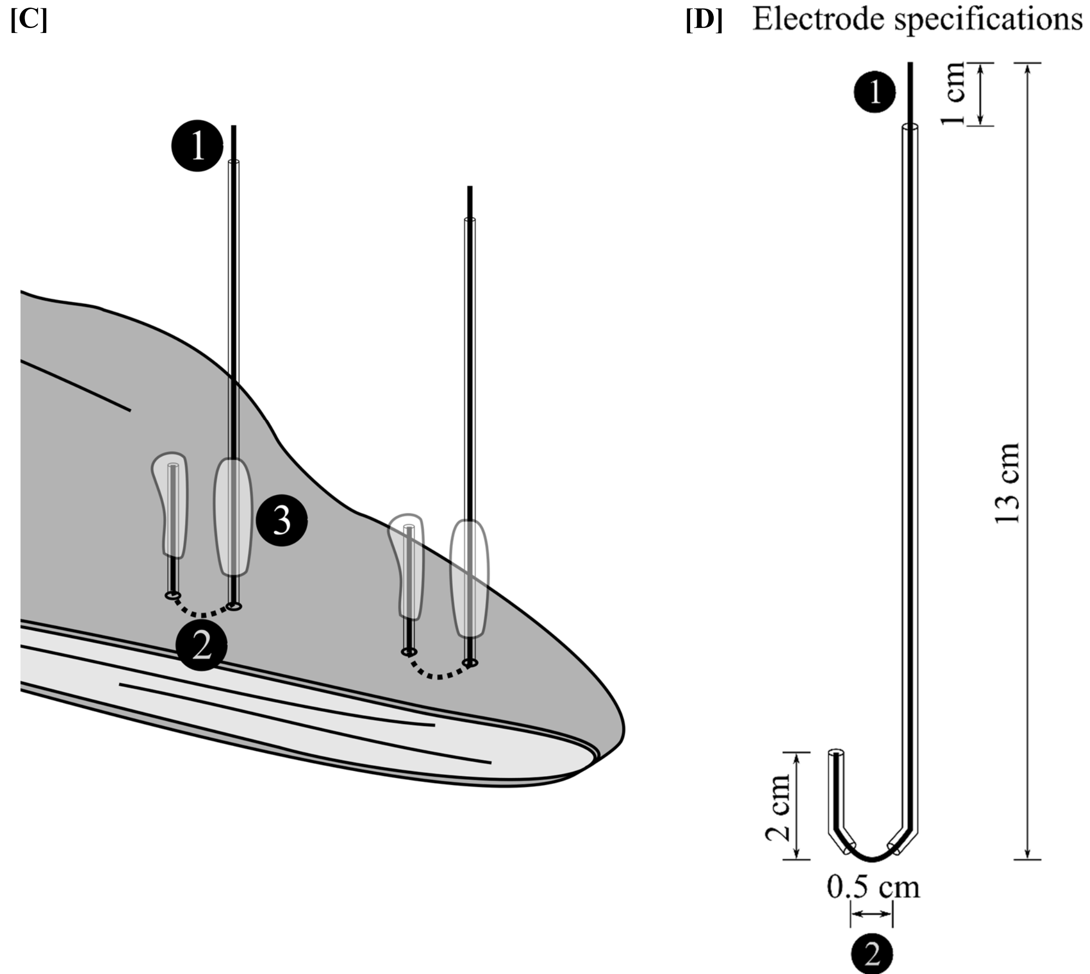



**Figure 2.5: Electrode implantation in *Aplysia*.**
**[A]** Sketch of a relaxed *Aplysia* with markings for electrode positioning.
**[B]** Image of the insertion of an electrode via a hypodermic needle. Before implantation, *Aplysia* were anaesthetised in iced artificial sea water. Hot glue was added after the hypodermic needle was extracted.
**[C]** Sketch of the tail of an *Aplysia* with two implanted electrodes. These were connected to the circuit on the long end ❶, where the isolation had been removed. The electrode was bent in the contact area, which was also bare and placed inside the animal tissue ❷. After insertion, the electrode was secured with a mass of hot glue ❸ on either side, where it left the animal.
**[D]** An electrode with length specifications. The bent part, i.e. the contact area ❷ and a small isolated part of the wire stayed inside the tissue so current could only flow inside the snail. The electrode was connected to the power source on the long end ❸.

Hot glue was put on either end to prevent the electrode from being pulled out accidentally (see figure 2.6).

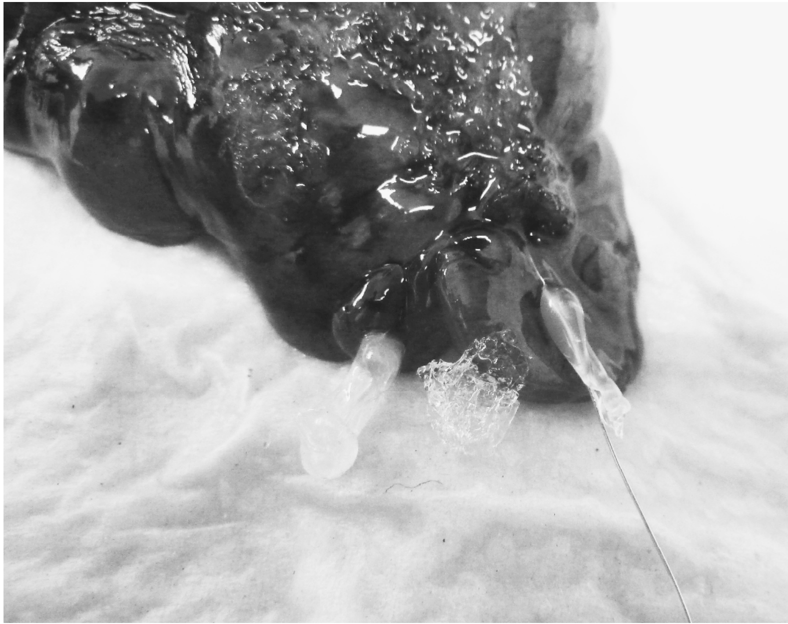


**Figure 2.6: Tail of an *Aplysia californica* with one implanted electrode.**
The wire was secured by means of hot glue on either side where it left the animal's skin.

In some cases, a piece of crepe tape folded around the long end of the wire instead of glue. This was to prevent the electrode from being accidentally extracted, if it got stuck e.g. in a corner of the housing box. For conditioning, the long end of the wire was connected to an electric circuit. In the cases where the long end of the wire had been secured with tape, this was removed before the pre-test to ensure visibility.

## 2.3. Equipment for training and testing

The workspace consisted of a water basin (1 m x 2 m x 20 cm), filled with fresh-water to a depth of 8 cm. The water constantly circulated through a cooler, to keep its temperature at 15 – 16 °C, i.e. equal to the tanks. Four white, robust boxes measuring 39.5 cm x 29.5 cm x 10 cm, were placed inside the basin. These were filled with aquarium water to a depth of about 8 cm and served as the experimental area for one *Aplysia* each. A picture of the workspace is shown in figure 2.7.

At least 19 h before the start of conditioning, 2 snails were placed inside two neighbouring boxes, to acclimatize to the new environment. Aeration was

provided by an air pump (LP-40, Resun, China), connected through tubes to lime-wood air diffusers (Red Sea, United Kingdom). A perforated plastic plate was used as a wall to form a separated compartment inside the experimental box. The air diffusers were attached inside this chamber, which measured about 3 cm x 29,5 cm x 10 cm.

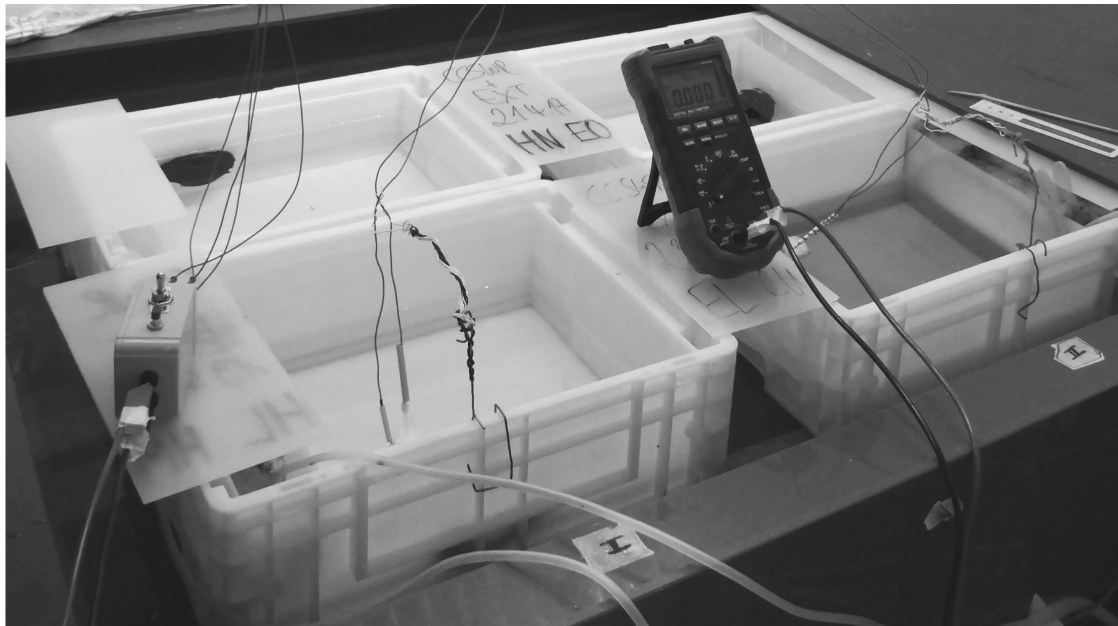


**Figure 2.7: Experimental workspace layout for classical conditioning.**
Throughout the experiment, subjects were kept within the white boxes in the experimental area. A pair of animals that was tested and trained together, was kept in a left and right box of one row.

### 2.3.1. Stimulus applications

#### 2.3.1.1. The conditioned stimulus

Wooden chop sticks (Fackelmann, Germany) were used to applicate the conditioned stimulus, these were bent near the top to form hooks of different sizes and angles in order to best reach the siphon in whatever position the snail would be at the time. In detail, the conditioned stimulus was conducted by inserting the chop stick into the siphon, touching the inner, upper part and pulling the stick upwards. Thereby the inner part of the siphon skin was touched on the whole way of roughly 1.5 cm to the top. Note the reaction of *Aplysia* in figure 2.8. This procedure lasted about 0.5 s and was performed similarly to Carew et al. (1981b).

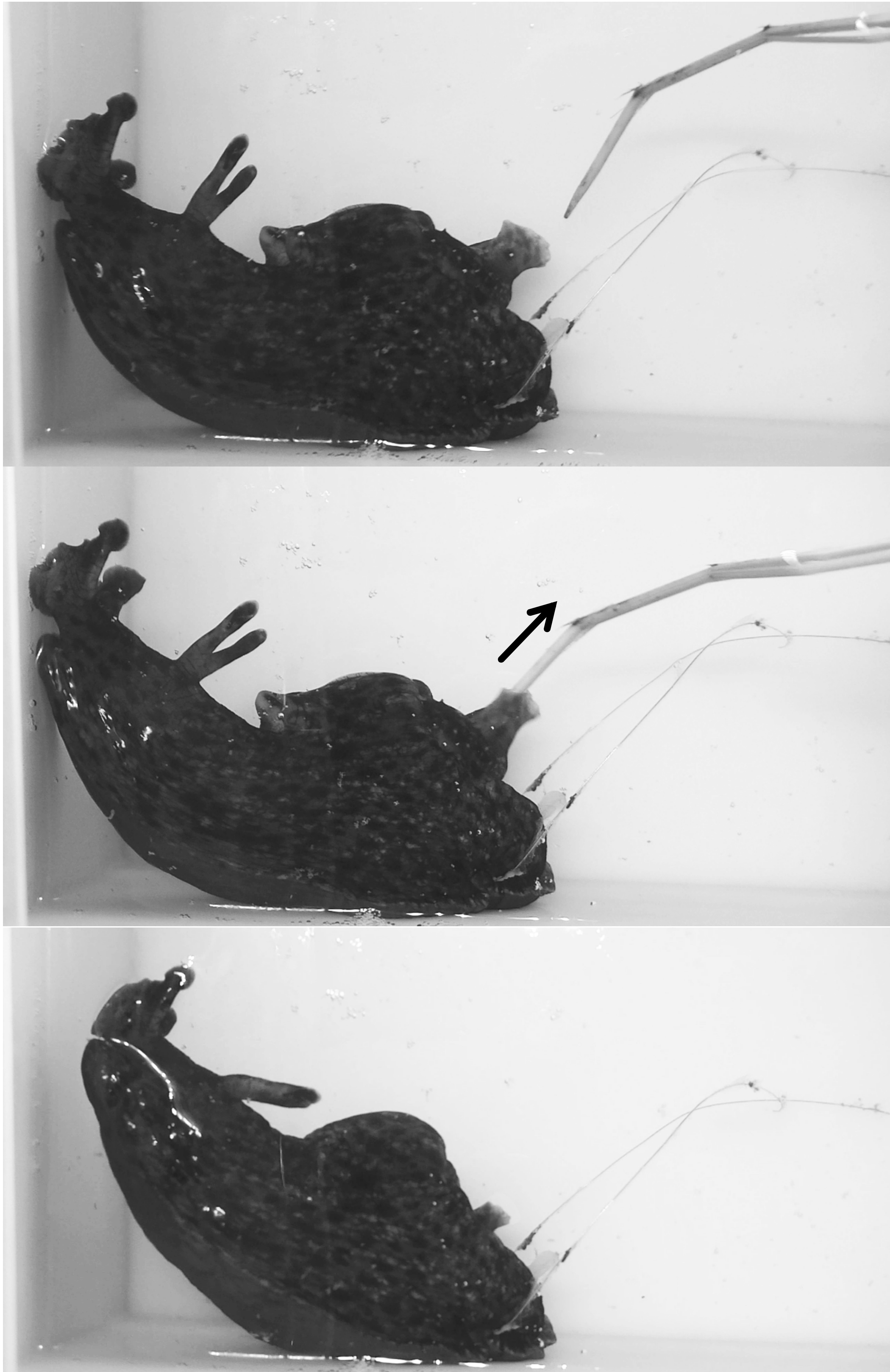


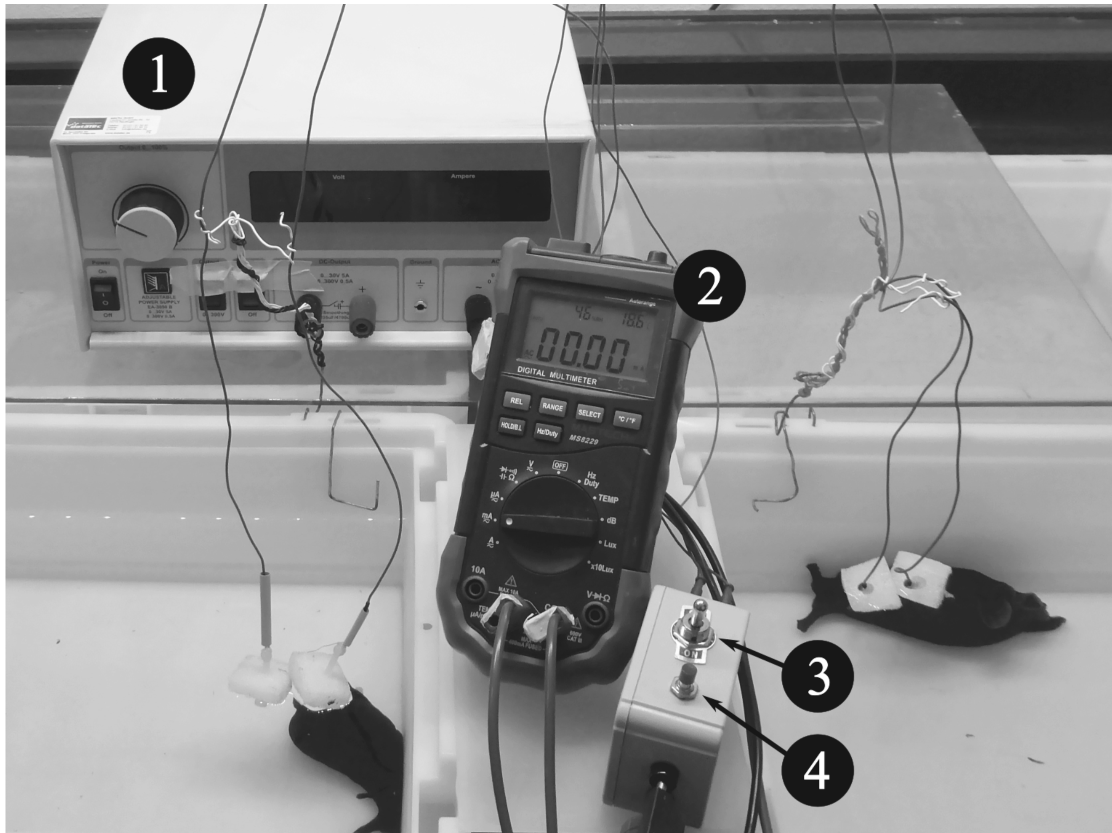
**Figure 2.8: Triggering of the siphon withdrawal reflex.**
A wooden chop stick was inserted into the siphon of *Aplysia* and in an upward and outward movement, the inner upper skin was stimulated. In naïve animals, this triggered a short withdrawal of the siphon.

### 2.3.1.2. The unconditioned stimulus

The unconditioned stimulus consisted of an electric shock of 1 s. Via implanted electrodes, in the mean 2.8 mA (1.0 – 3.4 mA) at 7 V / AC were applied. This intensity was determined in preliminary experiments as an intermediate strength, resulting in reliable conditioning. The electrodes were connected to an electric apparatus, which was designed for secure and reliable shock applications in two snails alternately. Figure 2.9 shows a picture of the components and the circuit diagram.

A power supply ① (EA-3051B, EA Elektro-Automatik, Germany) provided the current flow, which was measured by means of a digital multimeter ② (MS8229, MASTECH) during every single US application. Before every application, the respective animal was selected with a flip switch ③. Current flow was activated by manually holding a pressure switch ④. To stabilize the flow of current, a 1 kΩ series resistor ⑤ was included into the circuit.

**[A]**

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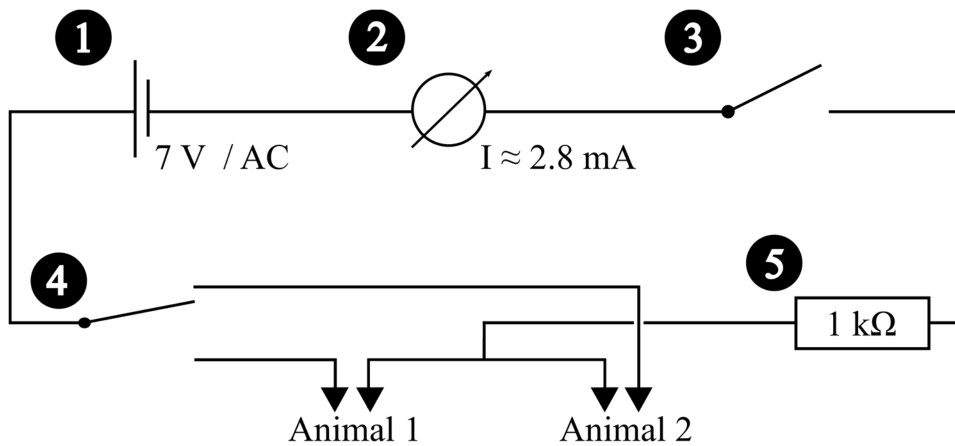



**Figure 2.9: Apparatus used for application of electric shocks and circuit diagram.**
The components of the apparatus:

- ❶ power supply
- ❷ digital amperemeter
- ❸ pressure switch
- ❹ flip switch
- ❺ resistor

The resistor was inside the small box that holds the two switches.

## 2.4. Experimental design

The main experiment of this work, was designed as a two-arm study, based on the first publication of classical conditioning of the siphon withdrawal reflex by Carew et al. (1981b). Animals of both groups were equally tested before training for naïve siphon withdrawal durations. This pre-test was scheduled at 14:45, classical conditioning started after a break of 30 min at 16:00. Tus, training ended before the natural resting phase of *Aplysia californica*, i.e. before the dark phase. The intervention group was sleep deprived for 6 hours following conditioning training, this group was named WAKE group. The control group, called SLEEP group, was left undisturbed after training. Retention of the conditioning memory was assessed at the 24h-test, which took place at 16:00 the following day. The 24h-test was followed by an extinction training in both groups. Extinction memory was tested in the 48h-test, at 16:00 on the third day of the experiment. Figure 2.10 provides an overview of the experimental design.

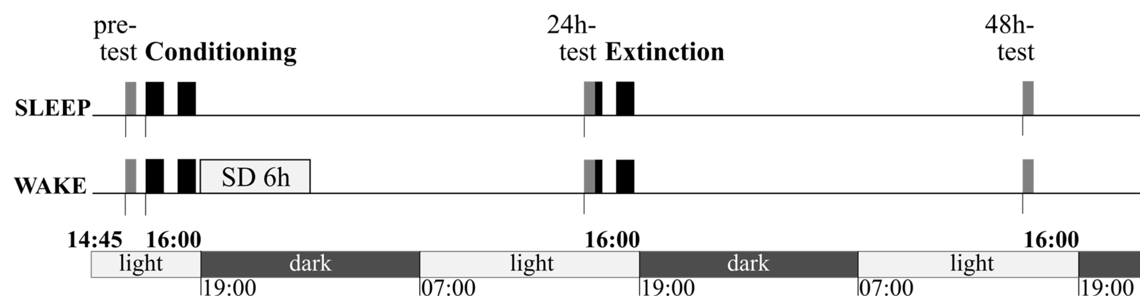



**Figure 2.10: Experimental design.**
Both experimental groups were tested three times: at the pre-, 24h- and 48h-test. Classical conditioning training was scheduled at 16:00 and followed by sleep deprivation of 6 hours in the WAKE group. After the 24h-memory test an extinction training was performed. Both trainings ended shortly before the dark phase. SLEEP = SLEEP group, WAKE = WAKE group, SD = sleep deprivation. n = 11 in both groups. Adapted from Thiede et al. (2021)

## 2.5. Experimental proceeding

### 2.5.1. Pre-test and classical conditioning

The pre-test consisted of the exclusive application of 7 tactile siphon stimulations (CS) to register the animal's untrained response. The number of applications was chosen to obtain enough trials with good visibility of the response. In preliminary experiments, the animals often started moving around after receiving the CS. For example, animals ascended the walls of the experimental boxes, making it impossible to see the siphon from outside the water.

After the end of the pre-test, the implanted electrodes were connected to the circuit. Conditioning started 35 minutes after the last pre-test trial (see figure 2.11 [A]). Training consisted of 20 pairings of tactile siphon stimulations and electric shocks, as earlier experiments had achieved classical conditioning after 15 – 30 trials (Carew et al. (1983), Carew et al. (1981b)). We adopted the scheme of Hawkins et al. (1989) and performed two separate training blocks of 10 pairings each, with an intermission of 45 min. The unconditioned stimulus followed the conditioned stimulus after 0.5 s. Only with this interval significant conditioning could be achieved in the experiment of Hawkins, Carew and Kandel (1986). An elongation of the inter stimulus interval of as little as 1 s had led to only marginal conditioning. In accordance with the setup of Carew et al. (1981b) and Hawkins et al. (1989), 5 min were chosen as the inter trial interval.

It was noted whether CS and US were performed correctly and if inking or opaline release occurred. With a pipette, ink and opaline were then removed from the water to clear the view. This occasionally delayed the following stimulus application. Further, current flow in mA as displayed by the multimeter was related, as well as any extraordinary event. After training, the water in the experimental boxes was exchanged with sea water from the aquaria.

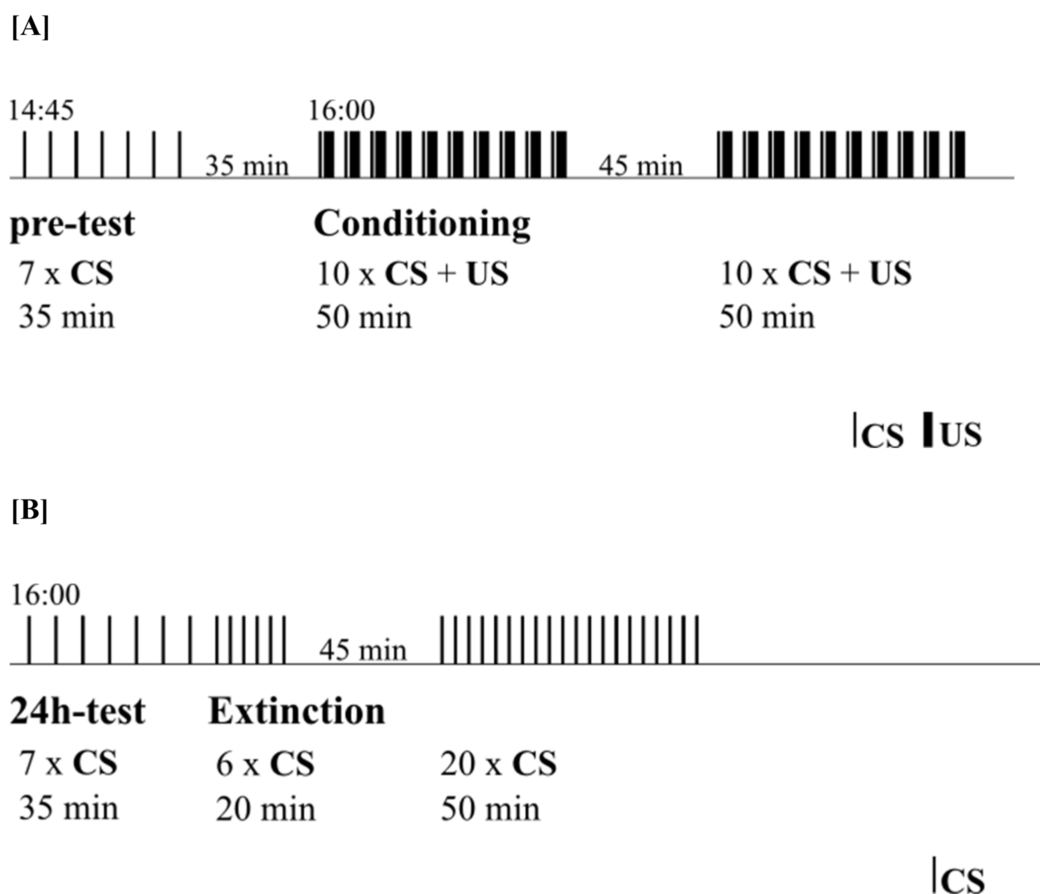



**Figure 2.11: Stimulus applications in the pre-test and conditioning.**
[A] The pre-test consisted in 7 tactile siphon stimulations with inter trial intervals of 5 min. Conditioning started at about 16:00, but at least 35 min after the last trial of the pre-test. There were two blocks of 10 paired stimulations, with an intermission of 45 min. The inter trial interval was 5 min, the inter stimulus interval 0.5 s.
[B] The 24h-test started at 16:00 and consisted in 7 tactile siphon stimulations with inter trial intervals of 5 min. Directly afterwards, 6 stimulations were applied as an extinction training, with breaks of 2.5 min. A second block with 20 stimulations (inter stimulus interval 2.5 min) followed after 45 min.
CS = conditioned stimulus, US = unconditioned stimulus. Adapted from Thiede et al. (2021)

### 2.5.2. 24h-test and extinction training

This retrieval test was conducted 24 hours after the beginning of the conditioning training, i.e. at 16:00 the following day. It was conducted identically to the pre-test with 7 trials of tactile siphon stimulation (see figure 2.11 [B]). After 24 hours, memory is considered long-term stored. As the 7 testing trials were not followed by a tail shock, they served at the same time as the first extinction trials.

For the rest of the extinction training, the inter trial interval was reduced by half, to 2.5 min. Like the acquisition, extinction training was performed in two blocks, with a break of 45 min. Taking together the 24h-test and the first block of extinction, 13 siphon stimulations were applied before the break. The second training block was of 20 CS in each animal, again the inter trial interval was 2.5 min.

### 2.5.3. 48h- and 72h-test

The outcome of the extinction training was assessed in another test, starting at 16:00 of the third experimental day. In 10 out of 12 WAKE group animals, a follow-up test 72 h after the beginning of conditioning training was additionally conducted. Both tests were performed in the same way as the pre-test.

### 2.5.4. Acoustic guiding of the stimulations

All steps of tests and training were guided by an audio file that was reproduced in the laboratory over loudspeakers. The correct timing of conditioned stimulus and unconditioned stimulus with an interval of only 0.5 s was hereby guaranteed. A beep sound of 1 s ensured that the pressure switch, which ran the current, was pressed for the correct duration. The instructions were repeated every 2.5 min. In that way, a pair of animals could be trained alternately, with an inter trial interval of 5 min in each animal. The audio was recorded and edited using audacity software (open source, Audacity Team) and a simple headset.

Figure 2.12 shows the transcript of the audio file (translated from German):

| | |
|---|---|
| 00:00:00 | one minute left |
| 00:00:30 | 30 seconds left |
| 00:45:00 | left / right |
| 00:50:00 | 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 |
| 01:00:00 | siphon |
| 01:00:50 | *(beep of 1 s)* |

**Figure 2.12: Transcript of the guiding audio for tests and trainings.**

The transcript shows the basic unit of instructions for application of one conditioned and unconditioned stimulus. The pattern was repeated 20 times every 2.5 min, providing guidance for 10 stimulations in 2 animals alternately. Left or right refers to the box position of the animal that was to be stimulated. The countdown was spoken rhythmically every second.

To the word “siphon”, the siphon was stimulated as the conditioned stimulus. During the duration of the beep tone, the electric shock as the unconditioned stimulus was applied. The audio file held 20 of the instruction units and was used for every test and training. In case of tests and extinction training, no unconditioned stimulus was performed to the beep sound.

## 2.6. Sleep deprivation

A method described by Vorster and Born (2017) was combined with a motivational approach, to deprive *Aplysia* from sleep. Like in their description, animals were removed from the surface they were attached to, if they stayed immobile for more than one minute. To unstick *Aplysia* from the substrate, a flexible plastic ruler was slid underneath the foot from head to the tail. Additionally, during the sleep deprivation period, snails were presented the odour of food. This was done to motivate the *Aplysia* to search for food and thus stay awake. One meal was therefore inserted into the aeration compartment of the experimental box. The flow of air from the aeration compartment to the rest of the experimental box caused water movement. In that way, food odour was distributed, but the animal was unable to reach the food. If necessary, a piece of ulva lactuca, clamped by a haemostat was held to the rhinophores or waved close to the tentacles. It was ensured that the snail was unable to swallow food. To enhance this effect and intrinsically motivate the snails, they were food deprived for 5 days before conditioning. Manual handling of the *Aplysia* could thereby be reduced. During the first hour after training, experimental animals were left undisturbed. In this period, little to no sleep was observed. Sleep deprivation ended at 1:00 am, resulting in a sleep deprivation period during the inactive phase of 6 h.

## 2.7. Behavioural analysis

### 2.7.1. Scoring of withdrawal durations

All tests and trainings were captured on video, to be analysed after the experiment. Advantages of this concept are the following: firstly, videos can be watched several times at different speed levels. The determination of the withdrawal duration is therefore more precise. Secondly, this method allows for verification through blind scoring. The start of the withdrawal duration was determined as the moment of deepest retraction. The end was defined as the time when the siphon had reappeared by 80%. All videos were manually scored by the author of this work. The results of the pre-, 24h- and 48h-test were verified by a blind scorer. Overall, scored siphon withdrawal durations were comparable, in case of a deviation of more than 30 s, the trial was re-scored.

For hands-free filming, two waterproof compact cameras (DMC-FT5, Panasonic Lumix) were positioned above the experimental area on two wall-hung jib-arms. These allowed close-up filming of the siphon movements. The software used to replay the videos was SMplayer (© Ricardo Villalba), which allows fast and slow motion and skipping back- and forwards. Figure 2.13 (below) shows the positions of the cameras.

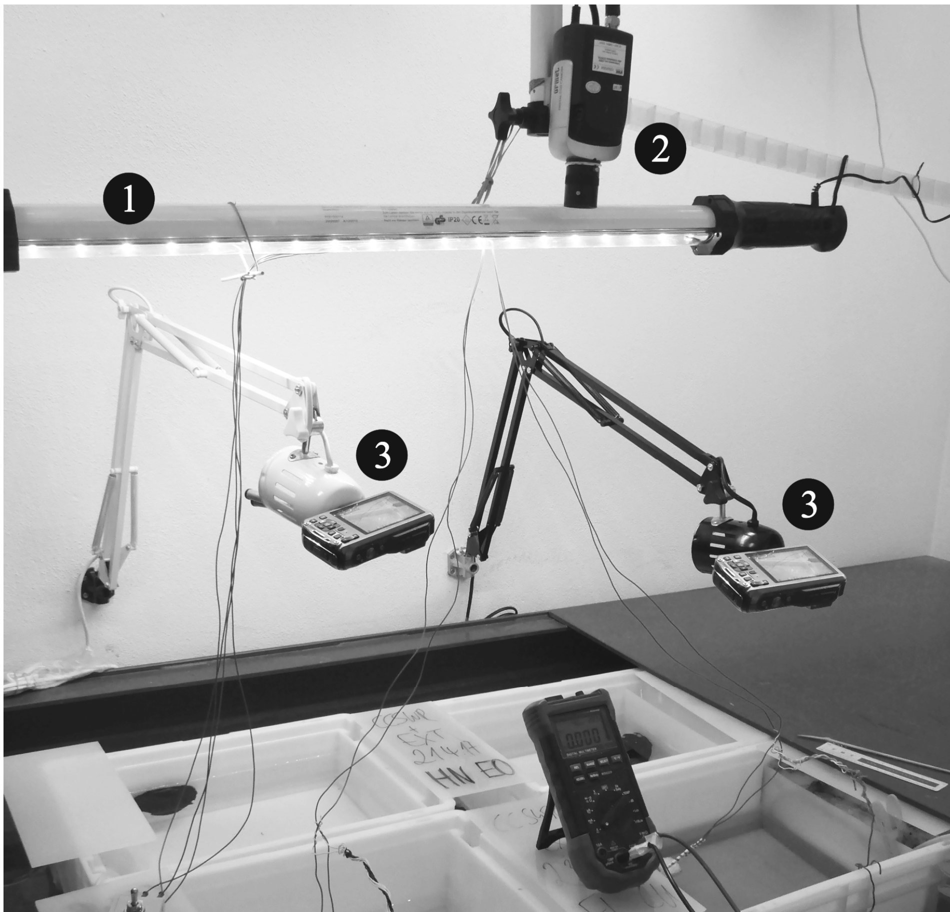



**Figure 2.13: Video recording setup**
The numbers in the picture refer to:
- ❶ additional LED lamp, for sufficient illumination during tests and training
- ❷ infrared camera, for day and night observation
- ❸ compact cameras, for close-up shoots of *Aplysia* siphon movements

## 2.7.2. Monitoring of sleep and wake activity

An infrared observation camera (DS 1092/004, URMET S.p.A., Italy) on a jib-arm attached to the ceiling captured the area of 4 white experimental boxes (figure 2.14, ❷), starting in the night prior to training. Infrared lamps (850 nm, SAL 35, B&S Technology, Germany) ensured visibility during night-time on the observatory video. A wall-hung lamp and a headlamp both equipped with a cut-off filter at about 600 nm (Primary red 106, Lee Filters, Andover, United Kingdom) provided visibility for the experimenter during sleep deprivation nights. The

photosensitivity of *Aplysia*'s eye and rhinophore in the red spectrum drops significantly above 600 – 660 nm (Waser (1968), Jacklet (1980)), thus the used light should not have an impact the circadian rhythm of *Aplysia*.

Scoring of the sleep and wake activity was executed using time lapse videos of the original observation videos with one minute shortened to one second. To track the snails' sleep and wake activity, all video observation material was first scored automatically by the software EthoVision XT 13 (Noldus Information Technology, Wageningen, Netherlands). In a second step, the scoring was verified by manual scoring.

## 2.8. Statistical analysis

Data was collected using Microsoft Excel (Microsoft Office 2016, Microsoft Corporation), calculations and diagrams were generated in Graphpad Prism (GraphPad Software, Inc.). For technical reasons, the maximum withdrawal duration that could be measured was 180 s. Any value above 180 s was counted as 200s. In all tests, 7 trials were performed. To determine the final score of an individual animal, the median was calculated of all available trial results. Individual results in the retention tests are shown as the difference to the pre-test value. Thereto, the median of 7 trials in the pre-test was subtracted from the median of 7 retention test trials. To compare withdrawal durations between two time points in one experimental group, paired t-tests were used. Gaussian distribution was explored using the D'Agostino & Pearson normality test. For differences of variance, an F test was performed. To compare the mean of the two groups at the same testing time, unpaired t-tests with Welch's correction were used. The two-way ANOVA with Sidak's multiple comparisons test was conducted to calculate the difference of the two experimental groups in two different time points. A one-way repeated measures ANOVA with Tukey's multiple comparisons test was used to compare three testing times in one experimental group. Correlations were calculated with the Pearson correlation. A p-value $< 0.05$ was considered significant.

### 2.8.1. A priori selection of animals excluded from statistical analysis

Even though conditions were kept stable throughout the whole study and all animas were treated in the same way, WAKE group animals showed significantly higher siphon withdrawal durations during acquisition. This elevation was correlated with higher scores in the 24h-test in the individual animals. To achieve comparability of the two experimental groups, those animals were excluded from the main analysis, which had exceeded the measurable limit of 180 s withdrawal duration in at least 5 of 20 acquisition trials. This was the case in 7 WAKE group animals, in all other animals the limit was only reached 3 times at the most.

Further, sleep quanta during the sleep deprivation period was controlled in both groups. SLEEP group animal had been immobile for 260 to 360 minutes. There was one outliner that had only 91 min of immobility. This was a value typically achieved by sleep deprivation in the WAKE group (range 0 to 147). Consequently, this animal was excluded from statistical calculations.

# 3. Results

## 3.1. Learning outcome

### 3.1.1. Classical conditioning

To investigate the influence of sleep on classical conditioning and extinction of the siphon withdrawal reflex in *Aplysia*, two groups of animals were trained. The interventional group (WAKE group) was sleep deprived for 6 hours after classical conditioning, the control group (SLEEP group) was left undisturbed.

#### 3.1.1.1. Individual siphon withdrawal durations in pre- and 24h-test

The means of baseline withdrawal duration (pre-test) of both groups (mean ± SD: SLEEP = 19.7 ± 6.0 s, WAKE 20.86 ± 3.7 s) were not significantly different (Figure 3.1). The two experimental groups are therefore considered comparable. Both groups showed longer siphon withdrawal durations 24 hours after training (mean ± SD: SLEEP 35.4 ± 18.4 s, WAKE 58.6 ± 53.1s). The pre-post differences were significant (mean of differences ± SD: SLEEP 15.7 ± 10.3 s, p = 0.0005; WAKE 37.7 ± 47.3 s, p = 0.0245, paired t-test).

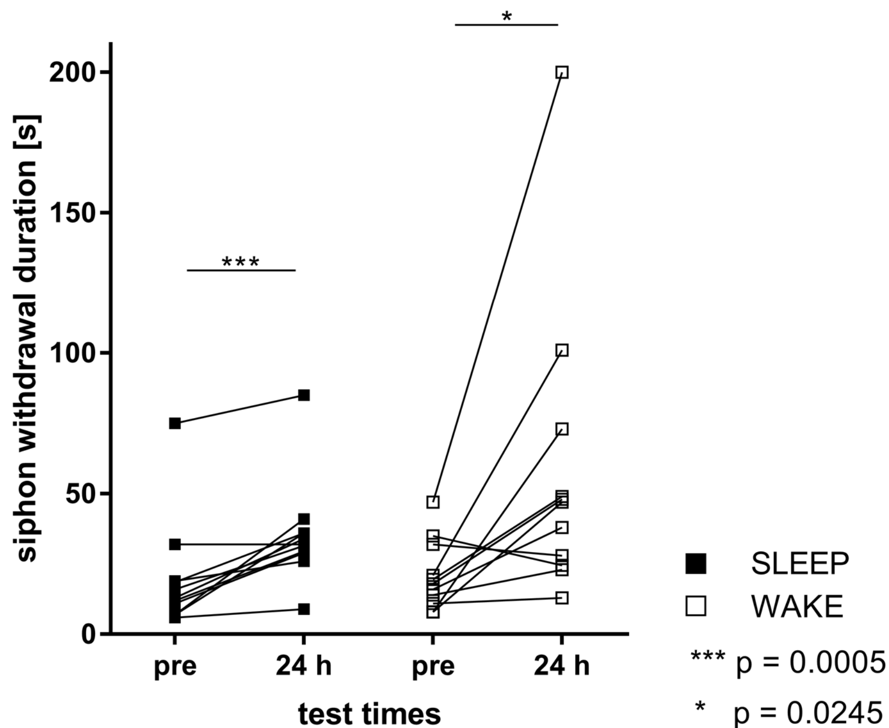



**Figure 3.1: Siphon withdrawal duration before and 24 hours after classical conditioning.**
Following the pre-test, animals were trained to associate a tactile stimulus of the siphon with an electric shock. Half of the animals were sleep deprived thereafter for 6 hours (WAKE), the others served as controls (SLEEP). The median of the 7 measured siphon withdrawal durations in each test, was used as the test result for the individual animal. Two paired t-tests were performed to analyse the group differences, p-values are two-tailed. Adapated from Thiede et al. (2021).

#### 3.1.1.2. Difference between pre- and 24h-test in siphon withdrawal duration

The difference of siphon withdrawal duration between 24h-test and pre-test value was considered the learning result of every individual animal. Similar learning was found in both groups (SLEEP 15.7 s ± 3.2 s, WAKE 37.7 s ± 14.3 s (mean ± SD), p = 0.16, unpaired t-test), see figure 3.2. Interestingly, the distribution of values was different for the two groups with a significant difference of variance (F

= 20.95, $p < 0.0001$, F-test). Data in this paragraph was published in Thiede et al. (2021).

#### 3.1.1.3. Difference between pre- and 48h-test in siphon withdrawal duration

Directly following the 24h-test, an extinction training with 26 trials of only the tactile siphon stimulus was performed. Contrary to the expectation, siphon withdrawal duration did not decrease but rather further increased when tested 48 hours after the initial training (figure 3.2). The difference to pre-test after 48 hours in siphon withdrawal durations is similar in the two groups (SLEEP 58.7 ± 21.2 s, WAKE 64.9 ± 17.2s (mean ± SEM), $p = 0.8229$, unpaired t-test). See also Thiede et al. (2021).

#### 3.1.1.4. Difference between pre-test and conditioning in withdrawal duration

Siphon withdrawal durations during conditioning were also registered during conditioning. The difference of siphon withdrawal duration from pre-test to conditioning reflects the enhanced siphon withdrawal due to the electric shock. To ensure both groups are comparable, this difference should be of similar height in SLEEP and WAKE group. The average values of the two groups are not significantly different (mean ± SEM: SLEEP 50.4 ± 11.9 s; WAKE 76.6 ± 8.4 s, $p = 0.0902$, unpaired t-test with Welch's correction), see figure 3.2. Data was published in Thiede et al. (2021).

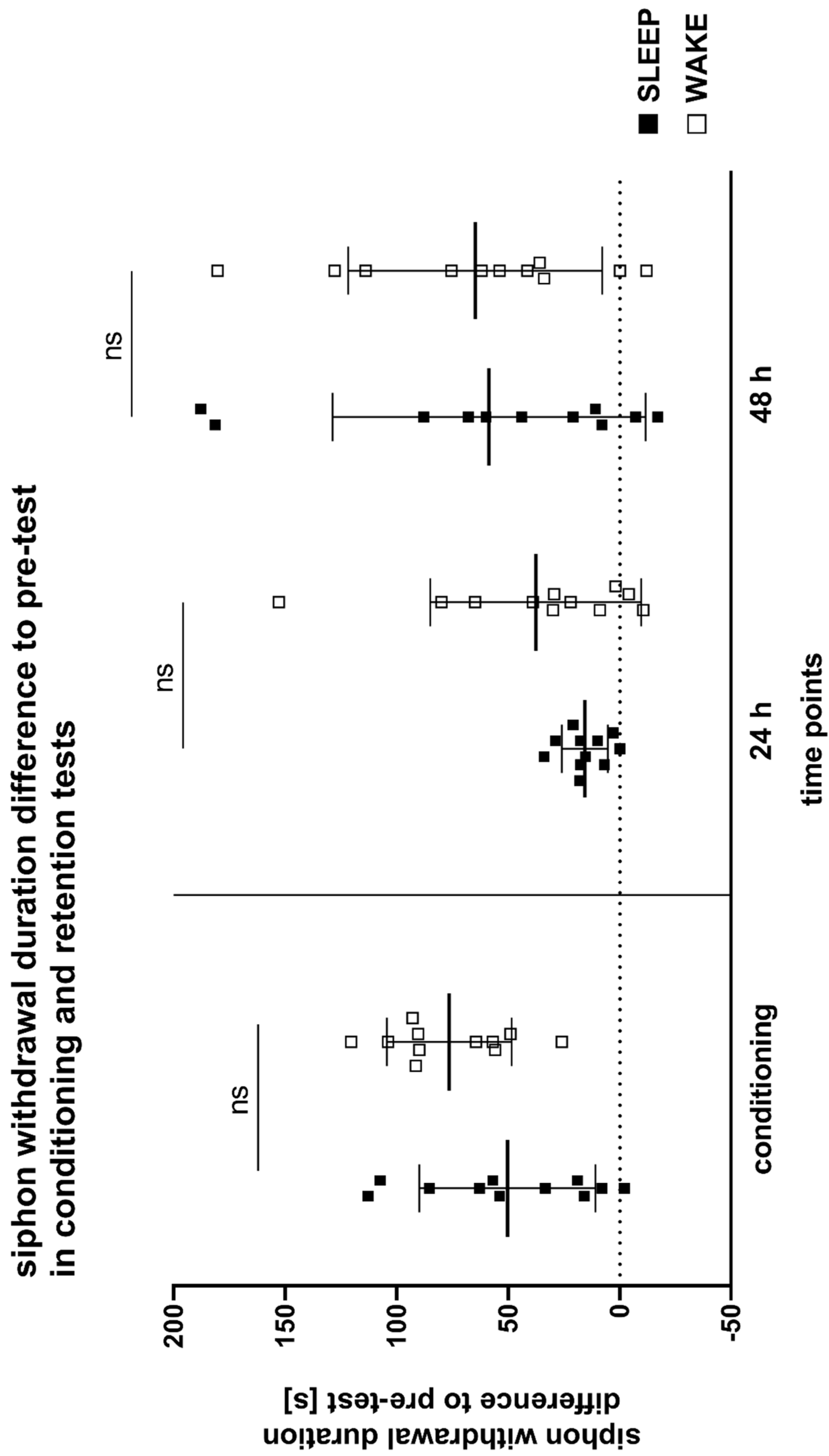



**Figure 3.2: Prolongation of withdrawal durations in acquisition, 24h- and 48h-test.**

**Figure 3.2: Prolongation of withdrawal durations in acquisition, 24h- and 48h-test.**
**Conditioning**: The difference median of 7 pre-test trials was subtracted of the median of 20 acquisition trials for each animal. In a condition trial, an electric shock to the tail was paired to the tactile siphon stimulation. The scatter dot plot shows the individual siphon withdrawal durations as a difference to pre-test and the group's average ± SD. Mean conditioning values are not significantly different in SLEEP (50.4 s) and WAKE group (76.6 s) ($p = 0.0902$, unpaired t-test and Welch's correction).
**24h- and 48h-test:** The median of 7 pre-test-trials was subtracted of the median 7 trials in 24h- or 48h-test. A test trial refers to the application of a tactile siphon stimulus without consequent electric shock. The scatter dot plot shows the individual siphon withdrawal durations compared to pre-test and the mean ± SD. The difference from 24h- to 48h-test result for each group is significant (two-way ANOVA and Sidak's multiple comparison test: $F(1,20) = 6.931$, $p = 0.016$).

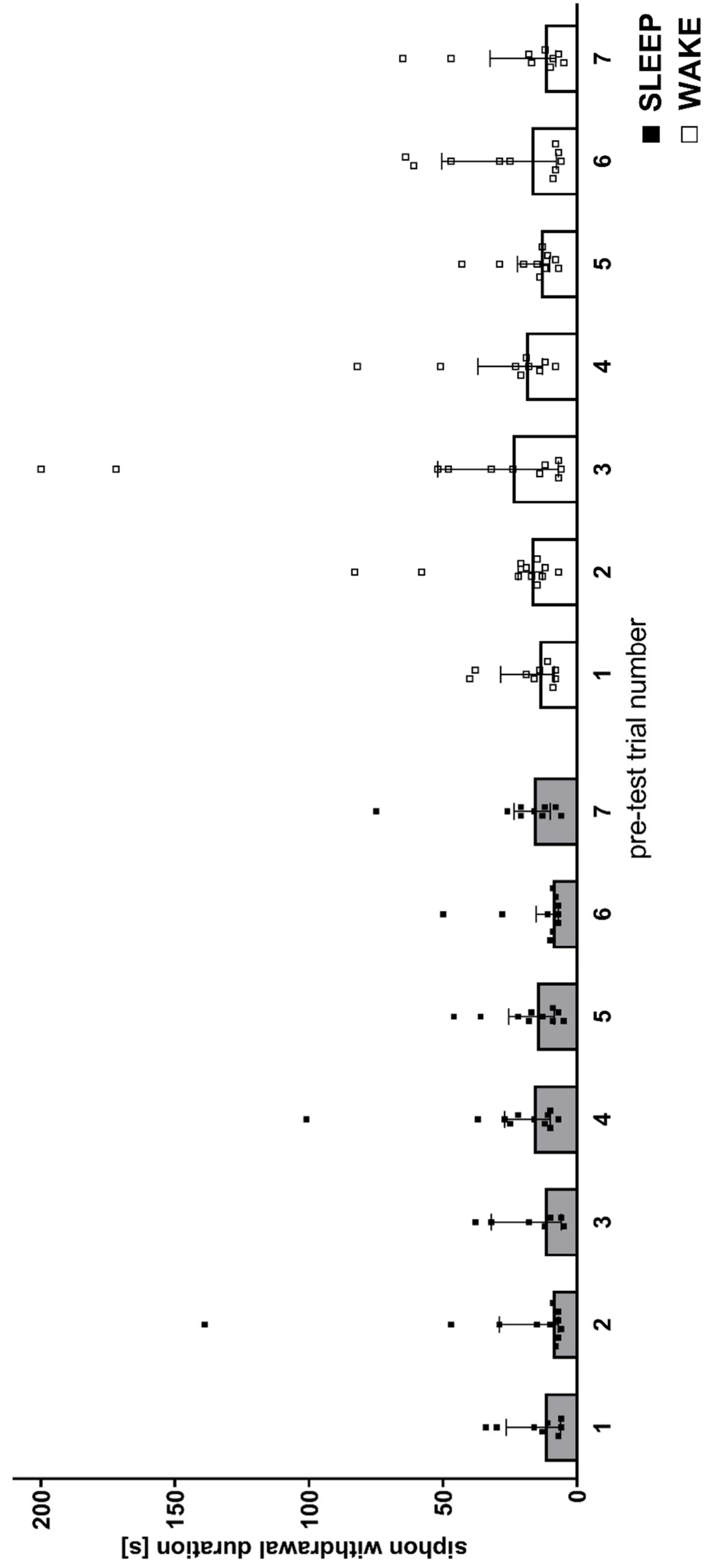
### 3.1.2. Extinction training

To measure the effect of extinction training, the result of the 24h-test and 48h-test (figure 3.2) were compared. Test result refers to the individual difference between the pre-test and 24h- or 48h-test siphon withdrawal duration. In neither of the groups, the extinction training reduced the siphon withdrawal duration (two-way ANOVA (group x time) with Sidak's multiple comparisons test): significant effect of time ($F_{(1,20)} = 6.931$, $p = 0.016$), no significant effect of group ($F_{(1,20)} = 0.6627$, $p = 0.4252$). Mean differences from 24h- to 48h-test ± SEM are: SLEEP 43.0 ± 20.7 s, WAKE 27.1 ± 16.8 s. These results were published in Thiede et al. (2021).

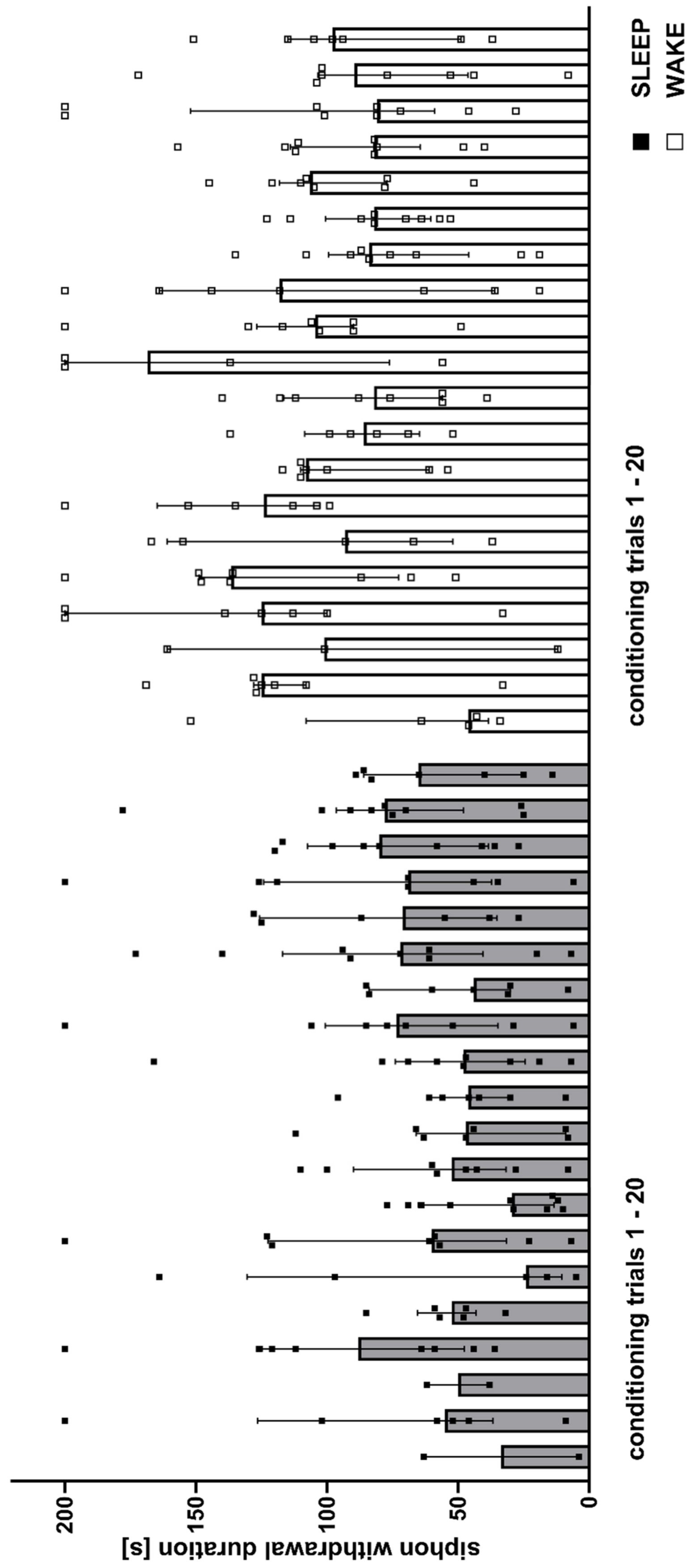
### 3.1.3. Trial means in conditioning and tests

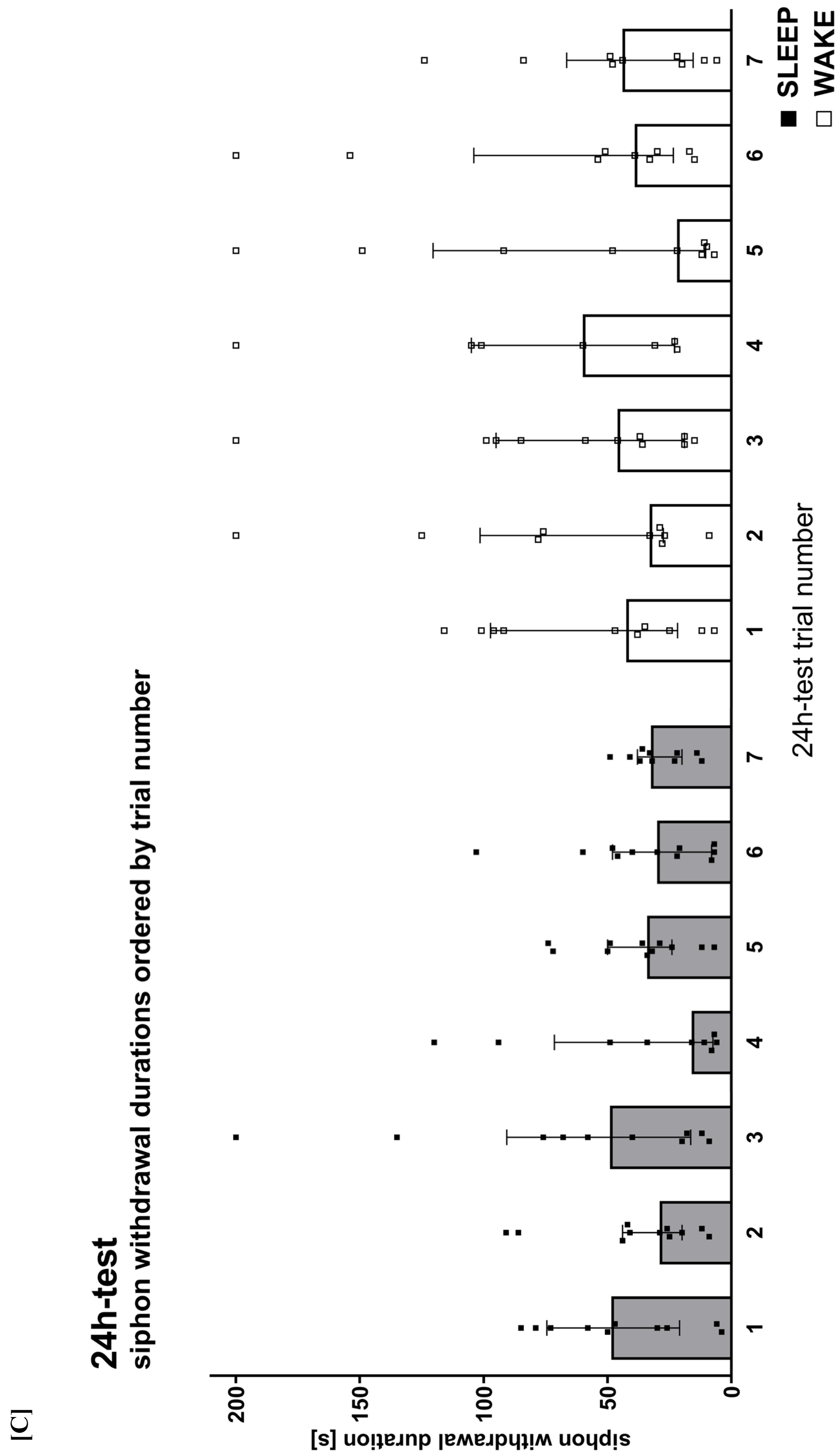
Further it was asked, if there were habituation or sensitization effects in the course of the trials of test and training. Figures 3.3 [A] – [D] show individual siphon withdrawal durations in every trial of all tests and training, ordered by groups. As siphon withdrawal durations are distributed non-Gaussian, the median was used as a representative value for each trial. No group effect of habituation or sensitization was found, as medians are not showing an upward or downward trend from trial 1 to trial 7 in tests and respectively trial 1 to 20 of the training.

[A]



[B]




[C]
24h-test
siphon withdrawal durations ordered by trial number
siphon withdrawal duration [s]
0
50
100
150
200
1
2
3
4
5
6
7
1
2
3
4
5
6
7
24h-test trial number
SLEEP
WAKE

[D]

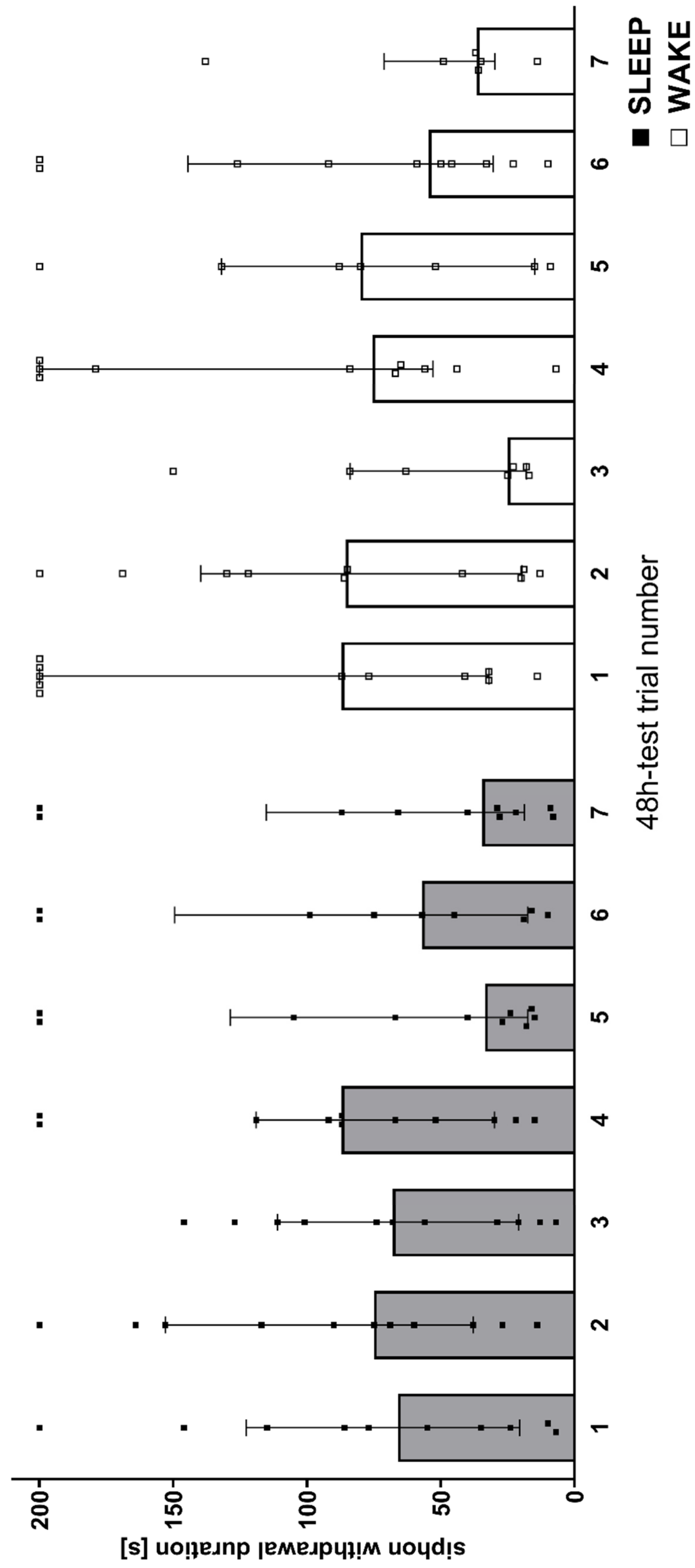


**Figure 3.3: [A] – [D]: Individual siphon withdrawal durations in trials of all tests and acquisition of all animals.**
Siphon withdrawal durations for each trial in SLEEP and WAKE group (n = 11 in either group). Bars show median ± interquartile range. Missing data points are due to technical difficulties in scoring of the siphon withdrawal duration. The pre-test was conducted 30 min before acquisition and consisted of 7 tactile stimulations of the siphon. After training, WAKE group animals were sleep deprived for 6 hours, SLEEP group animals left undisturbed. 24 hours after training, the siphon withdrawal was tested 7 times with tactile stimulation as in pre-test. The 24h-test was followed immediately by extinction training. A 48h-test with the same specifications as pre- and 24h-test (7 trials only tactile stimulation, 5 min inter trial stimulation) was performed.

### 3.1.4. 72h follow-up test

To gain an impression of the persistence of the conditioning memory, 16 WAKE group animals were tested a third time. 72 hours after training, the mean of the differences to pre-test withdrawal duration in the 72h-test was of 64.5 ± 51.7 s (mean ± SD). This is in between the 24h-value (48.7 ± 40.2 s) and 48h-test (85.1 ± 64.6 s). Withdrawal durations after 24, 48 and 72 hours are not significantly different from one another (one-way RM ANOVA: $F = 2.997$, $p = 0.0675$). Memory for conditioning was thus still existent 72 hours after training.

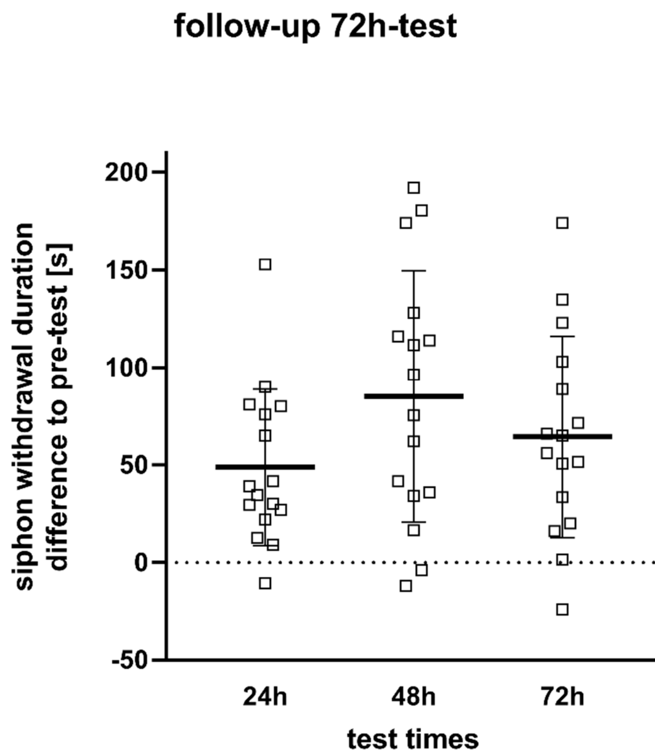



**Figure 3.4: Siphon withdrawal duration as difference to pre-test of *Aplysia* before and 24 hours after classical conditioning and 24 and 48 hours after extinction training.** Following the pre-test, animals were trained to associate a tactile stimulus with an electric shock in a classical conditioning protocol. WAKE group animals were sleep deprived for 6 hours after training. All animals were tested for siphon withdrawal duration 24 hours after acquisition. The 24h-test was followed by an extinction training of 26 tactile stimulations, the effect of which was controlled in the 48h-test. Another retrieval test was performed 24 hours thereafter (72h-test). The graph shows individual results as the difference of siphon withdrawal duration in 72h-test to pre-test, lines are mean values ± standard deviation, n = 16. The means are not significantly different from each other (1way RM ANOVA: $F = 2.997$, $p = 0.0675$).

## 3.2. Sleep quanta of experimental groups

Animals were monitored by means of infrared cameras throughout the whole experiment. Sleep durations scored as described in Material and Methods. Conditioning training ended shortly before the beginning of the dark phase, in tests and training, animals were considered awake. During the first half of the 12-hour dark phase, WAKE group animals were deprived from sleep by presentation

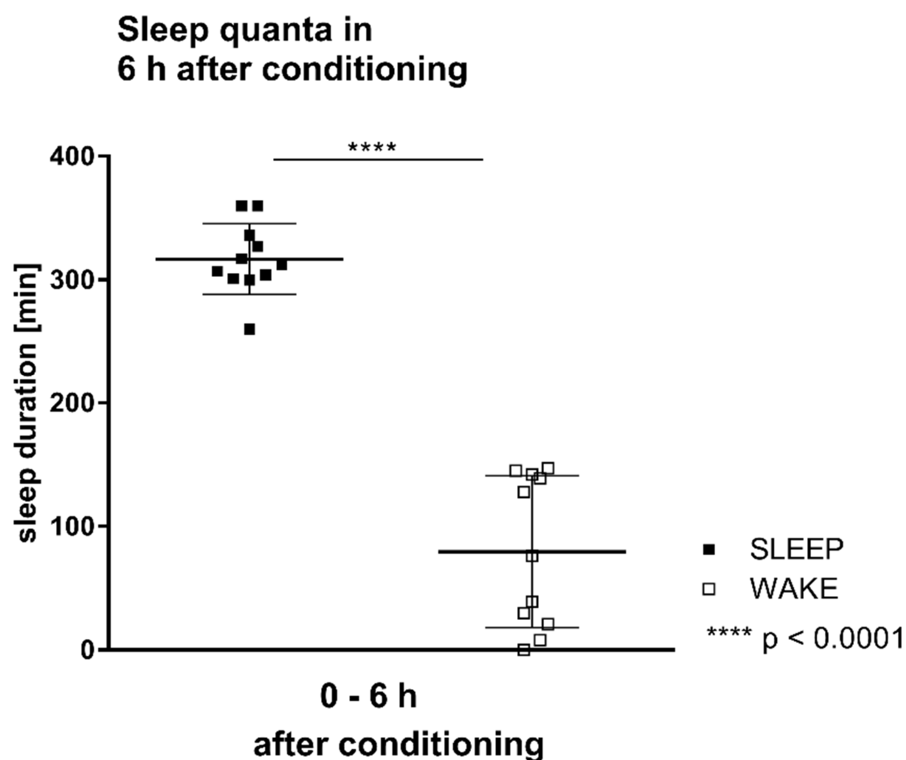
of food odour and by soft tactile handling. Figure 3.5 [B] gives an overview of the total sleep duration in 24 hours after conditioning.

### 3.2.1. Sleep quanta in 6 and 24 hours after training

Within 6 hours after training, on average SLEEP group animals slept 317 min (range: 260 to 360 min), WAKE group animals 80 min (range: 0 to 147).

Further, sleep after acquisition was assessed until the 24h-test and still there was a significant difference (p = 0.0002) for the two groups: SLEEP group animals were asleep for 829 ± 102 min (mean ± SD), WAKE group animals for 514 ± 206 min (mean ± SD). The results in this paragraph were published in Thiede et al. (2021).

[A]



[B]

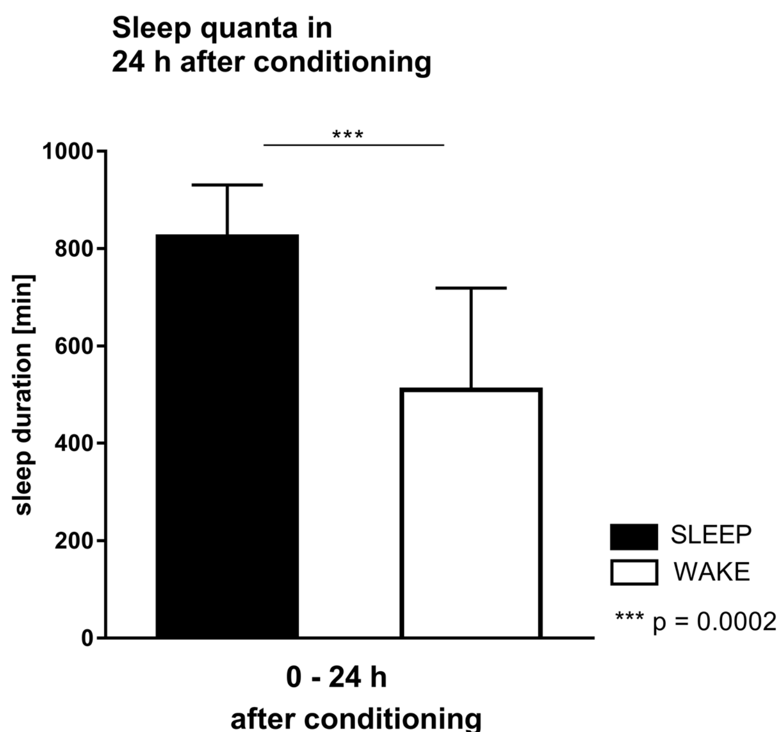



**Figure 3.5: Sleep duration 6 and 24 hours after conditioning training in sleep deprived and control group animals.**
Animals were considered asleep if not moving for 5 minutes, n = 11 for both groups. After classical conditioning training, SLEEP group animals were sleep deprived by presentation of food odour and gentle manual handling for 6 hours.
**[A]** The difference in sleep between SLEEP and WAKE group in 6 hours after training was 237 min ($F = 4.56$, $p < 0.0001$). Lines show the mean, whiskers the standard deviation.
**[B]** For the 24-hour period, the difference of sleep quanta between the two groups was 315 min ($F = 4.073$, $p = 0.0002$). Bars show mean, whiskers the standard deviation.

### 3.2.2. Sleep quanta in 48 hours after training

During the second night following sleep deprivation, WAKE group animals did not show a sleep rebound (see figure 3.6). The average sleep duration in the SLEEP group was 826 min (range: 401 to 1190 min) and in the WAKE group 797 min (range: 540 to 961 min).

The cumulative sleep duration in 48 hours was still significantly different between the two groups (SLEEP 1626 ± 222 min, WAKE 1339 ± 318 min; $p = 0.0235$, unpaired t-test). Data in this paragraph was published in Thiede et al. (2021).

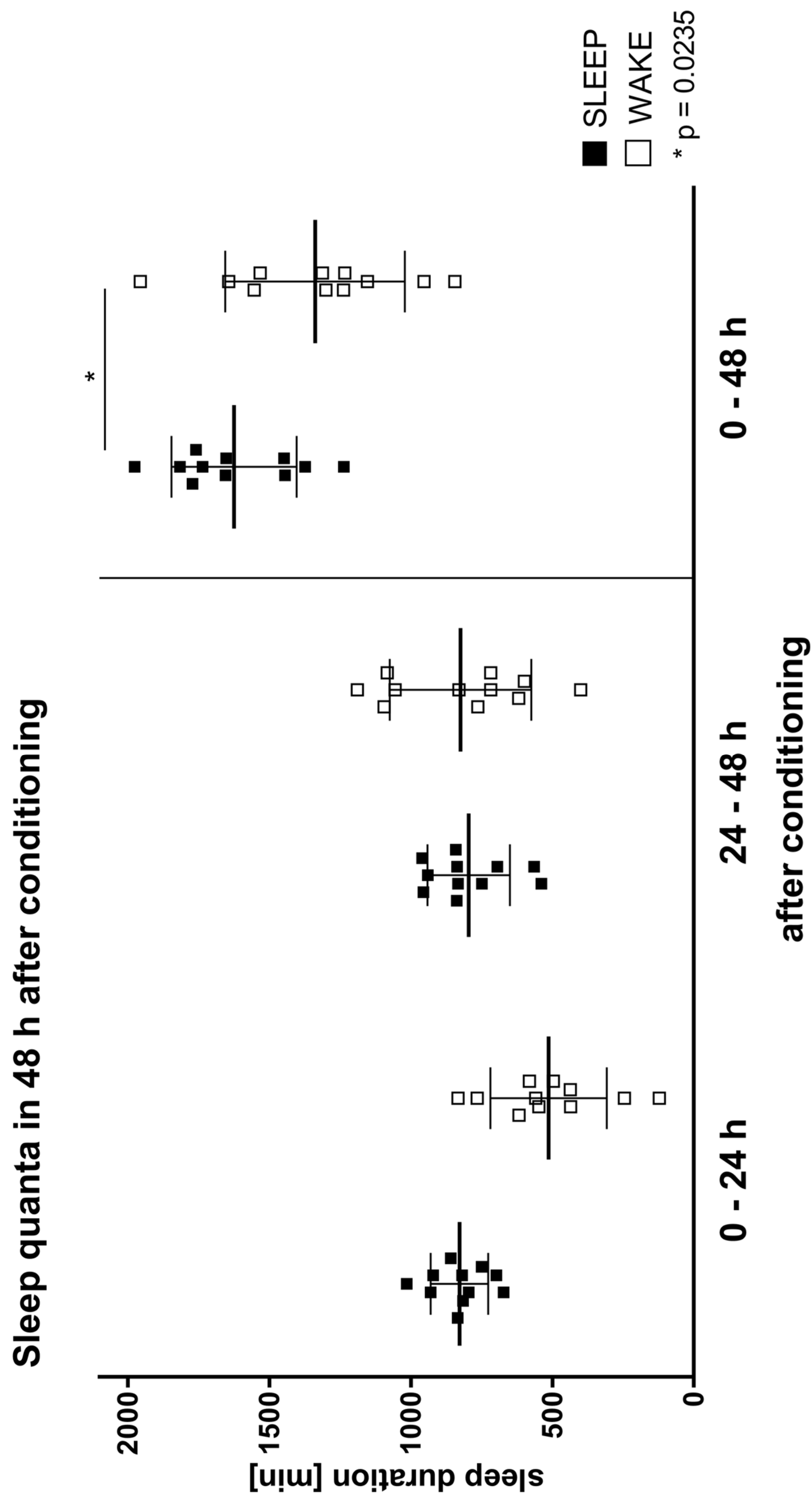



**Figure 3.6: Sleep duration in 48 hours after conditioning training, in sleep deprived and control group animals.**

**Figure 3.6: Sleep duration in 48 hours after conditioning training, in sleep deprived and control group animals.**
Animals were considered asleep if not moving for 5 minutes, n = 11 for both groups. After classical conditioning training, SLEEP group animals were sleep deprived by presentation of food odour and gentle manual handling for 6 hours. Before the 24 – 48-hour time span an extinction training was performed. The 0 - 48 h values are the sum of 0 – 24 h and 24 to 48 h for each group respectively. Lines show mean ± standard deviation. An unpaired t-test between mean sleep durations in 0 – 48 h for SLEEP group (1626 min) and WAKE group (1339 min) shows a significant difference with p = 0.0235 (F-test: F = 2.056).

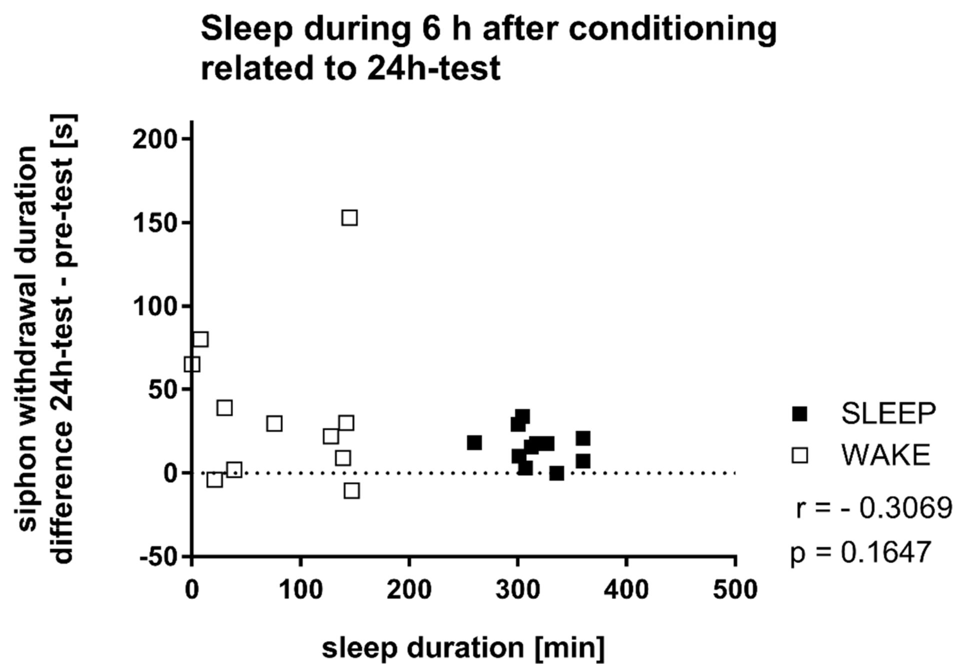
## 3.3. Correlation of sleep quanta and learning

To further assess the influence of sleep on classical conditioning, the sleep durations after training were correlated with the prolongation in siphon withdrawal in the retrieval tests after 24 and 48 hours (figure 3.7). Prolongation in siphon withdrawal refers to the difference in withdrawal duration between pre-test and 24h- or 48h-test. The time span of 6 hours after acquisition represents the period of sleep deprivation for the WAKE group animals.

### 3.3.1. Sleep during 6 h after training and siphon withdrawal durations

There is no significant correlation of the amount of sleep during 6 hours after training and the increase of siphon withdrawal duration after 24 hours (r = - 0.3069, p = 0.1647), Thiede et al. (2021). Sleep in 6 hours after training was neither significantly correlated to the increase in siphon withdrawal duration after 48 hours (r = - 0.01467, p = 0.9483). Data points for both groups are plotted in the same graphs, black and white indicate SLEEP and WAKE group (figure 3.7).

[A]




[B]

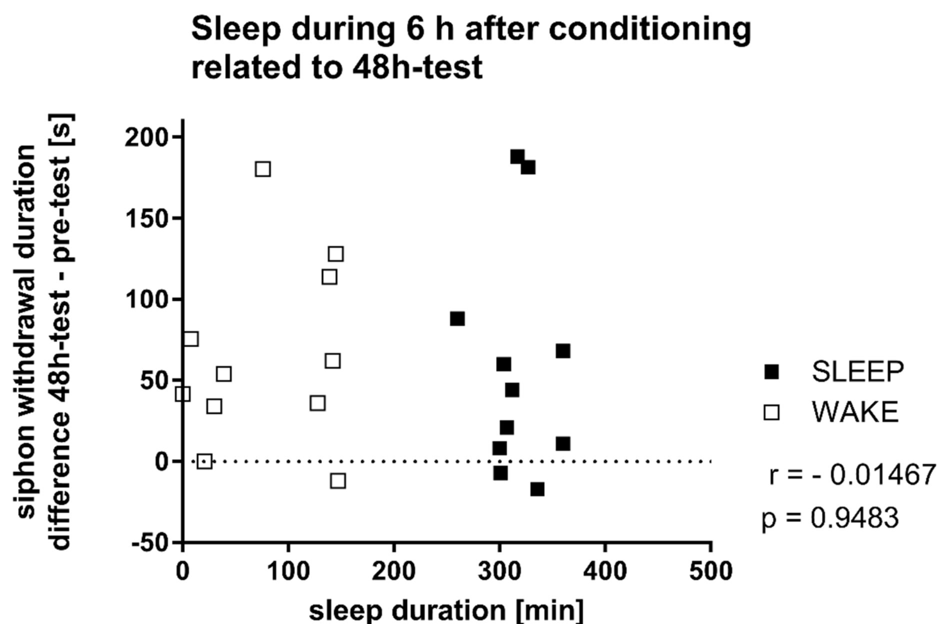


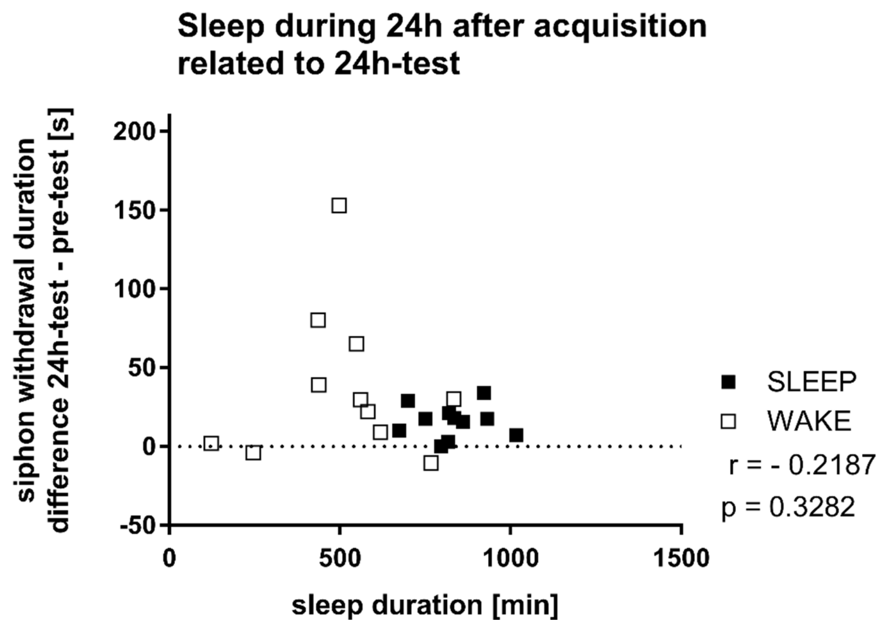

**Figure 3.7: Correlation of sleep duration during 6 hours after training and behavioural outcome.**

Animals were tested for baseline siphon withdrawal duration before classical conditioning. After 24 hours, a retrieval test was performed, followed by an extinction training. The effect of the latter was tested in the 48h-test. Test values for individual animals are the median of 7 trials, in this figure depicted as the difference to pre-test. N = 11 in both groups. Sleep duration in 6 hours after training is correlated with **[A]** the 24h-increase in siphon withdrawal duration (Pearson's r = - 0.3069, p = 0.1647), adapted from Thiede et al. (2021) **[B]** the 48h-increase in siphon withdrawal duration (Pearson's r = - 0.01467, p = 0.9483).

### 3.3.2. Sleep in 24 hours and siphon withdrawal durations

Sleep in 24 hours after acquisition was insignificantly correlated to the difference of siphon withdrawal duration to pre-test after 24 hours (r = - 0.2187 and p = 0.3282). Neither was the increase in withdrawal duration after 48 hours was significantly correlated to sleep in 48 hours after training (r = 0.07184, p = 0.7507).

**[A]**




**[B]**

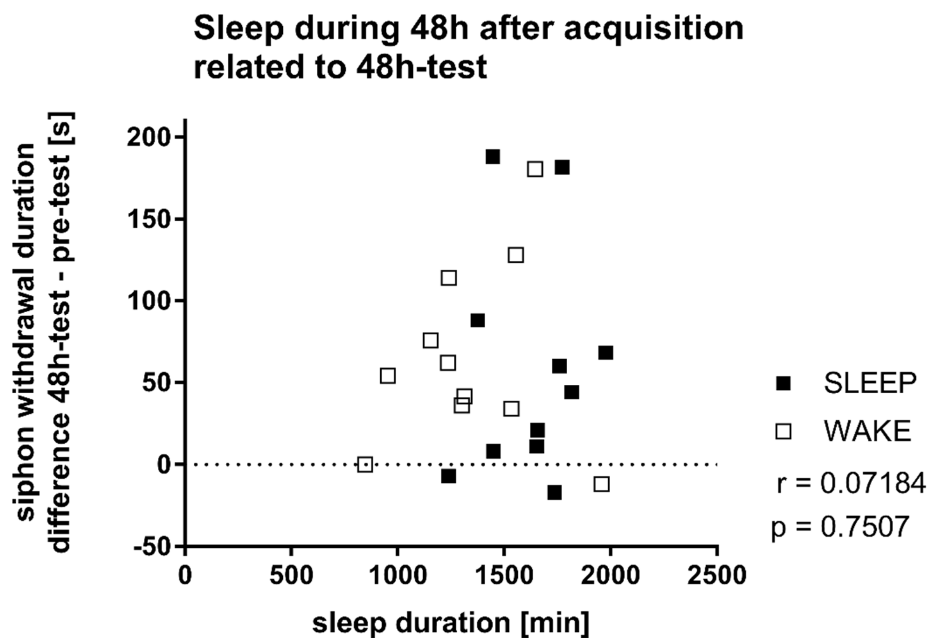


**Figure 3.8: Correlation of sleep duration in 6 hours after training and behavioural outcome.**
Before conditioning, animals were tested for baseline siphon withdrawal duration. After 24 hours, a retrieval test was performed, followed by an extinction training of 23. The effect of the latter was tested 24 hours after extinction training in the 48h-test. Test values for individual animals are the median of 7 trials and in this figure depicted as the difference to pre-test. N = 11 in both groups. The correlation of sleep duration in 12 hours after training with **[A]** the 24h-increase in siphon withdrawal duration (Pearson's r = - 0.2187, p = 0.3282), **[B]** the 48h-increase in siphon withdrawal duration (Pearson's r = 0.07184, p = 0.7507) was not significant in either case.

## 3.4. Supplemental Results

### 3.4.1. Preliminary experiments

#### 3.4.1.1. Determination of shock intensity

To ascertain the lowest possible current flow for the unconditioned stimulus, which would reliably induce learning in classical conditioning, two preliminary experiments were performed. The setup was equal to that of the main experiment. A pre-test for baseline siphon withdrawal was followed by classical conditioning. After 24 hours, a retention test was conducted.

The two preliminary experiments served to compare different electric shocks in conditioning, thus either 4.5 V / 2.5 mA or 10 V / 4.1 mA were applied as the unconditioned stimulus. The learning effect of both groups is shown in table 1. Group sizes are n = 11 for 4.5 V and n = 15 for 10 V. Both the 4.5 V and 10 V group show significant increase in siphon withdrawal duration after 24 hours (unpaired t-test: p = 0.0280 (4.5 V), p = 0.0021 (10 V)). Yet the difference in duration from pre- to 24h-test in most of the animals trained with 4.5 V was quite small (median = 8 s). In the 10 V sample the difference was more pronounced (median = 31 s). The median change in siphon withdrawal duration (24h - pre) was significantly different between the two groups (Mann-Whitney test, p = 0.0182).

**Table 3.1: Behavioural outcome 24 hours after classical conditioning of *Aplysia californica* trained with either 4.5 V or 10 V.**

Test results for individual animals are the median of 7 trials. The group mean pre-test score was compared in a t-test to the mean 24h-test score. Further, the pre-test score was subtracted from the pre-test score for every individual animal, the median of these differences is noted in the last column.

| | mean pre-test [s] | mean 24h-test [s] | p-value t-test: pre vs 24h-test | median difference 24h-test – pre-test [s] |
|---|---|---|---|---|
| 4.5 V | 14.6 | 30.2 | 0.0280 | 8 |
| ± SD / range | ± 13.3 | ± 21.8 | | - 5 to 50 |
| 10 V | 17.6 | 76.4 | 0.0021 | 31 |
| ± SD / range | ± 14.7 | ± 61.2 | | 0.5 to 191 |

#### 3.4.1.2. Current flow for different voltage

In the experimental setup, only voltage could be manipulated. Table 2 gives an overview of the resulting current flow. Groups were of n = 11 (4.5 V), n = 15 (10 V) and n = 22 (7 V, main experiment).

**Table 3.2: Resulting current for different voltages applied to *Aplysia californica* via implanted electrodes.**
Electrodes were stainless steel wires of 125, 200 or 250 µm diameter, the diameter had no influence on resulting current (see supplements), implanted on the left side of animals' tail region with approximately 4 cm distance from one another.

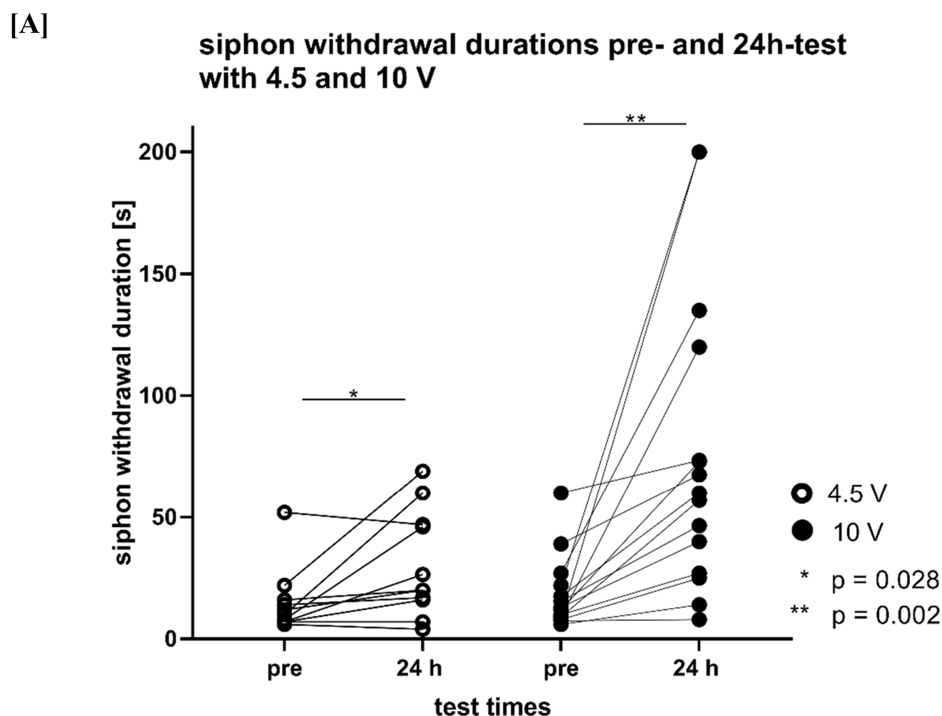
| applied voltage | 4.5 V (preliminary) | 10 V (preliminary) | 7 V (main experiment) |
|---|---|---|---|
| mean current in mA | 2.5 | 4.1 | 2.8 |
| range | 2.2 – 2.9 | 1.9 – 5.7 | 1.0 – 3.4 |

#### 3.4.1.3. Learning with different stimulus intensities

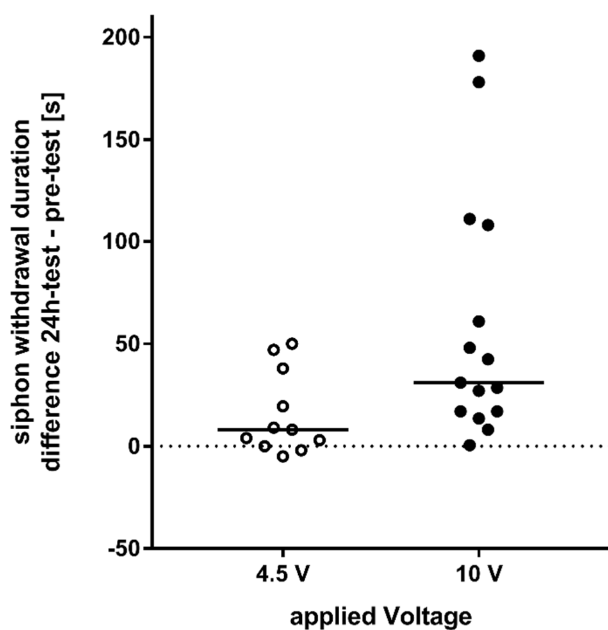
In the two preliminary experiments, both groups had learned as they exhibited prolonged siphon withdrawal durations in the 24h-test. The mean pre-test withdrawal duration was 14.6 ± 13.3 s in the 4.5 V group and 17.6 ± 14.7 s in the 10 V group (mean ± SD). In the 24h-test, mean withdrawal durations were 30.2 ± 21.8 s in the 4.5 V group and 76.4 ± 61.2 s in the 10 V group.

The individual difference in siphon withdrawal duration between 24h- and pre-test was more pronounced in the 10 V group. The median difference in the 4.5 V group was 8 s and 31 s in the 10 V group.

**[A]**




**[B]**



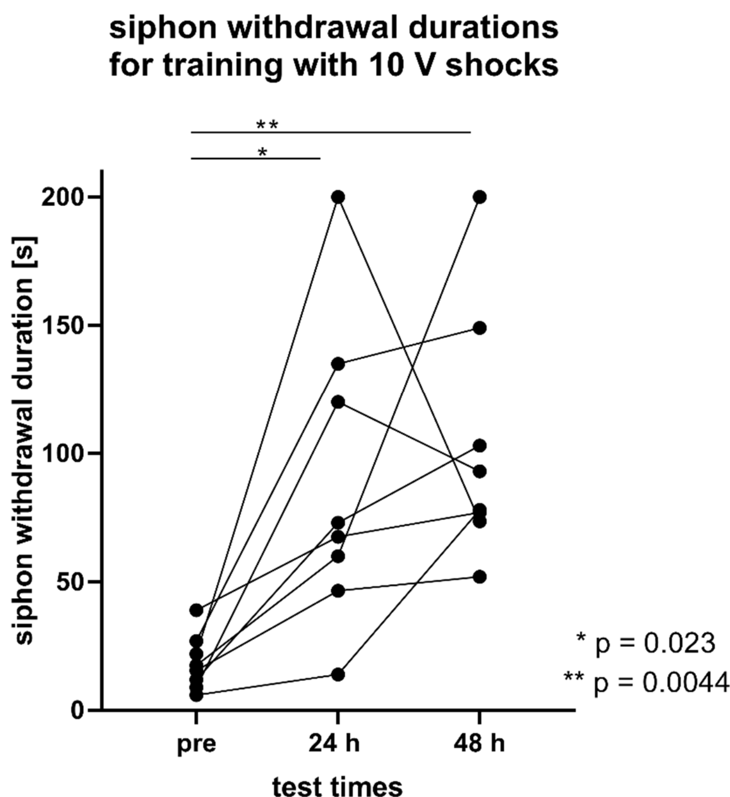

**Supplemental figure 1: Learning effect of classical conditioning of the siphon withdrawal reflex with 4.5 and 10 V shocks.**
**[A]** The pre- and 24h-test results of each animal were compared. Two paired T-tests show significant differences in both groups (p values are two-tailed).
**[B]** The difference between 24h- and pre-test value for each animal of both groups is depicted as a dot. The group median is represented by the horizontal line and is of 8 in the 4.5 V group and of 31 in the 10 V group.

### 3.4.1.4. Preliminary extinction training

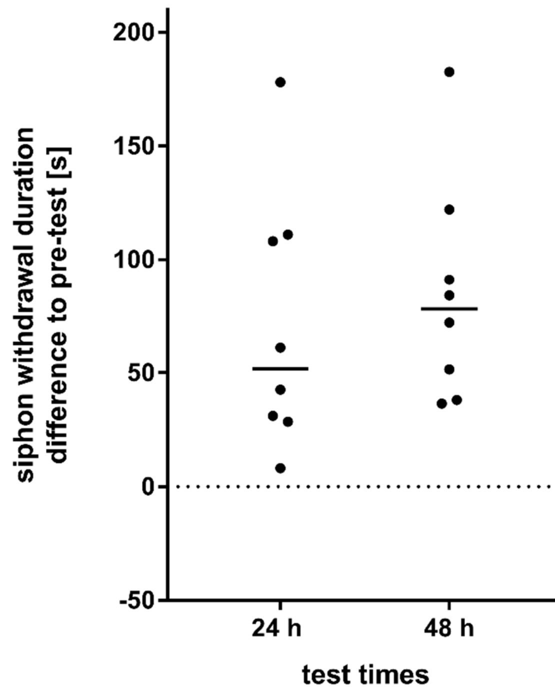
An extinction training was performed with 8 *Aplysia* of the 10 V / 4.1 mA group after the 24h-test, following the same instructions as the main experiment (see Material and Methods). The siphon withdrawal duration was then measured 48 hours after conditioning, to assess extinction effect (see supplemental figure 2 [A]). Instead of a reduction, most animals showed a further increase in absolute siphon withdrawal duration from 24h - to 48h-test i.e. after extinction training. This prolongation did not reach a significant level. The change in behaviour was significant from pre- to 24h-test ($p = 0.023$) and from pre- to 48h-test ($p = 0.0044$) (one-way repeated measures ANOVA, $F = 8.722$, $p = 0.0076$ with Tukey's multiple comparisons test).

The individual difference of siphon withdrawal durations after 24 and 48 hours to the naïve pre-test withdrawal duration was higher after 48 hours with a median of 78 s (range: 36.5 – 182.5 s) than after 24 hours, where the median was of 52 s (range: 8 – 178 s), see supplemental figure 2 [B].

[A]



**[B]**

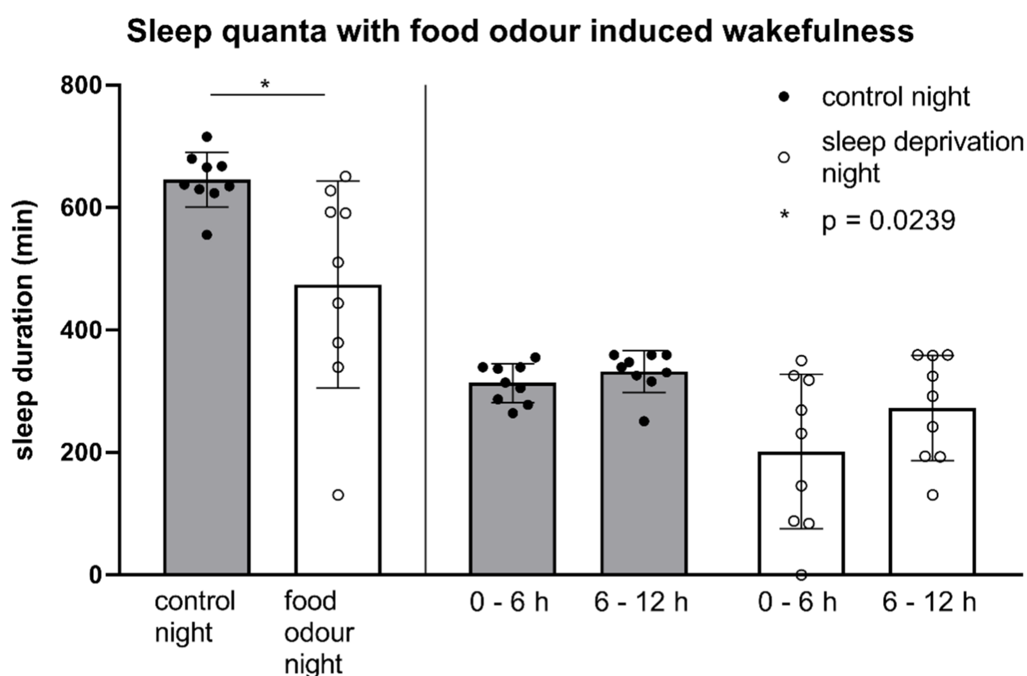



**Supplemental figure 2: Behavioural outcome of classical conditioning of the siphon withdrawal reflex with a tail shock of 10 V / 3.9 mA in *Aplysia californica*.**
All tests consisted of 7 trials of tactile siphon stimulation and the median of these was used as the resulting score. Animals were trained after the pre-test with 20 pairings of tactile siphon stimulations and 10 V / 4.1 mA / AC tail shock. After the 24h-test, an extinction training of 26 tactile siphon stimulations was performed. 24 hours thereafter, the siphon withdrawal duration was reassessed.
**[A]** Data points are the scores of each animal in each test. A one-way ANOVA for differences in pre- and 24h-test-scores reveals significance ($F = 8.722$, $p = 0.0076$), Tukey's multiple comparisons test confirms significant differences between pre- and 24h-test ($p = 0.023$) and pre- and 48h-test ($p = 0.0044$).
**[B]** Siphon withdrawal durations are demonstrated as the difference of the 24h-test score to pre-test score, for 48h-test respectively. In a paired t-test, the results of 24h- and 48h-test proved to be not significantly different ($p = 0.6249$).

Derived from these results, an intermediate voltage of 7 V was used in the main experiment.

### 3.4.1.5. Evaluation of sleep deprivation method

To physically disturb animals as little as possible during sleep deprivation, the effect of food odour as a wakefulness inducing stimulus was tested. Of 9 *Aplysia* that had not been fed for at least 5 days, sleep quanta in two different nights were compared. In one night, food odour was present in the water for the first 6 hours of the dark phase without the possibility to eat. The control night was a random night without any further intervention. In the night with food odour, the quantity of sleep was significantly reduced from 646 ± 45 min to 474 ± 169 min (mean ± SD, paired t-test, p = 0.0239). However, the range of sleep quanta in food odour night was very broad, reaching from only 131 min of sleep to 651 min.

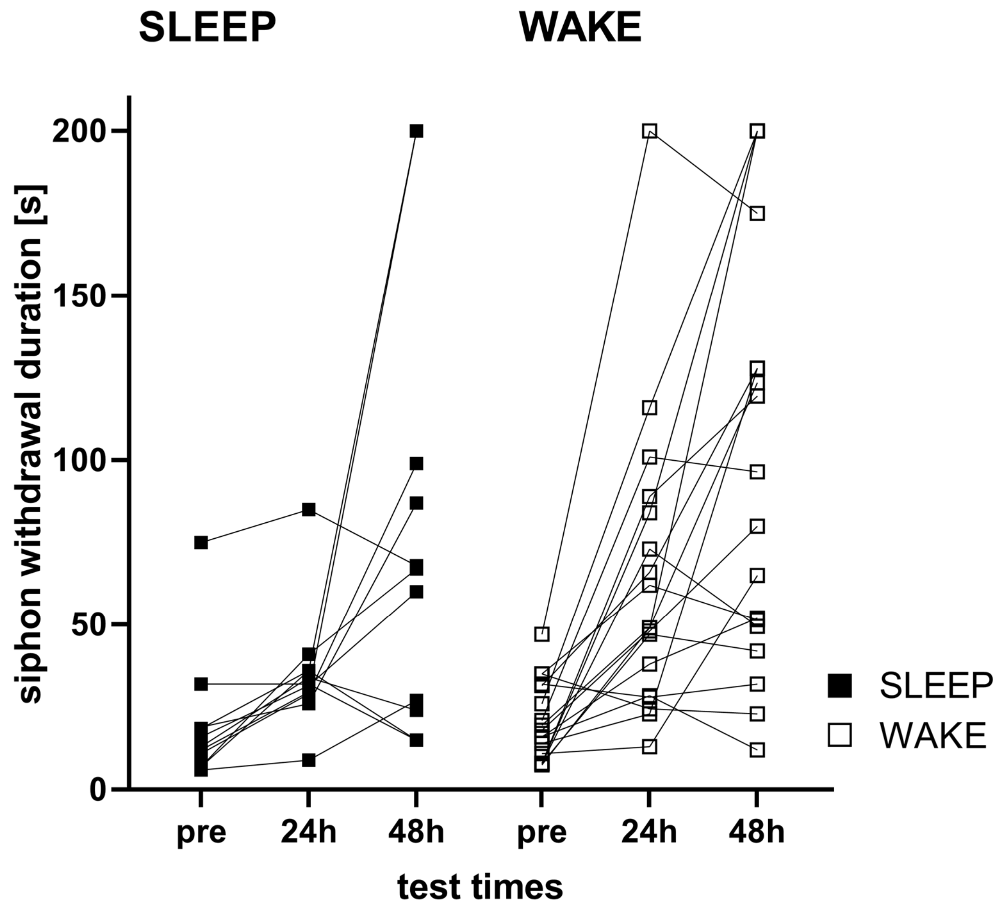



**Supplemental figure 3: Sleep duration of 9 animals during night with food odour and control night.**
Animals had been deprived from food for at least 5 days and for about 6 hours. In the sleep deprivation night, food odour was presented by inserting one meal into the aeration compartment of the experimental boxes, animals could not reach the food. They were more active in those nights when food odour was present (paired t-test, p = 0.0239). On the left, the full nights are shown, on the right, the same nights are split into the first and the second half. The difference in sleep quanta in the first and second half of the sleep deprivation night was not significant (paired t-test, p = 0.1796).

## 3.4.2. Supplemental analysis of the main experiment

### 3.4.2.1. Individual siphon withdrawal durations in all tests

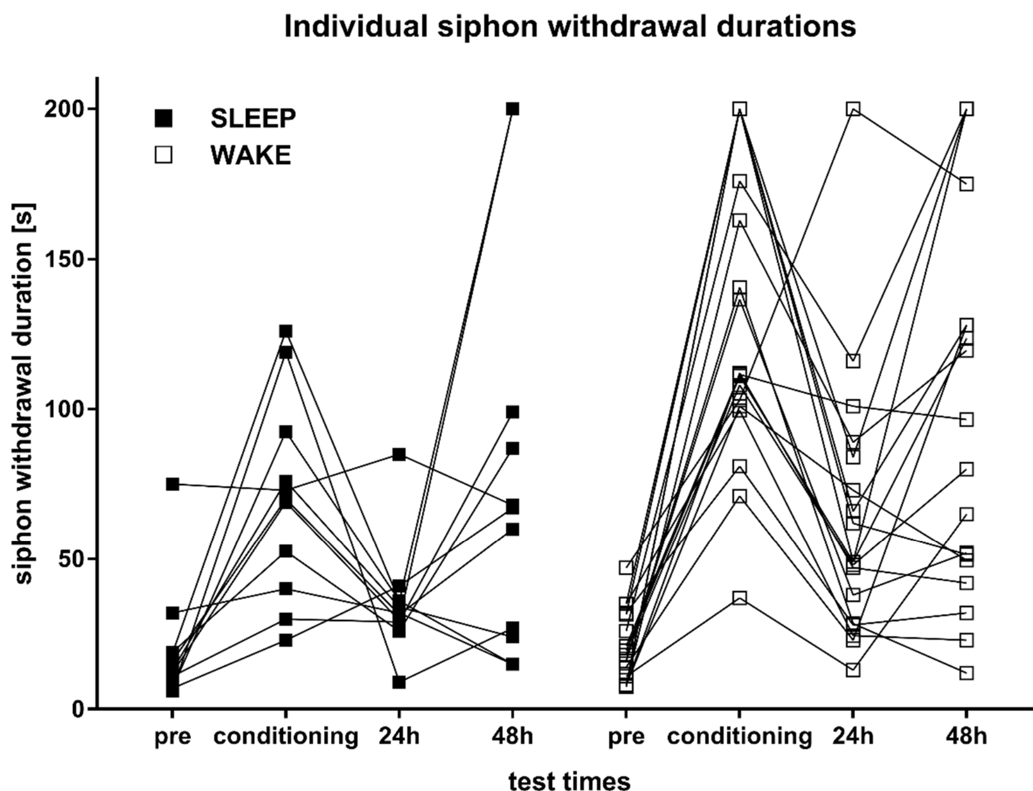
A clear learning effect was found 24 hours after conditioning that persisted after 48 hours.




**Supplemental figure 4: Siphon withdrawal durations at three testing points.**
Each line represents one experimental animal. Data points are of all animals, including those that were excluded from statistical analysis. Following the pre-test, animals were trained to associate a tactile stimulus of the siphon with an electric shock. 12 animals were sleep deprived thereafter for 6 hours (WAKE), the others served as controls (SLEEP). After the 24h-test, 26 extinction trials of only tactile stimulations were performed. Each test consisted of 7 trials, of only tactile stimulations. The median of the measured siphon withdrawal durations was used as the test result for the individual animal.

### 3.4.2.2. Individual siphon withdrawal durations in conditioning and tests

Siphon withdrawal durations were also measured at conditioning where they were significantly prolonged. Supplemental figure 5 allows for comparison of individual withdrawal durations at conditioning to the duration at all tests.



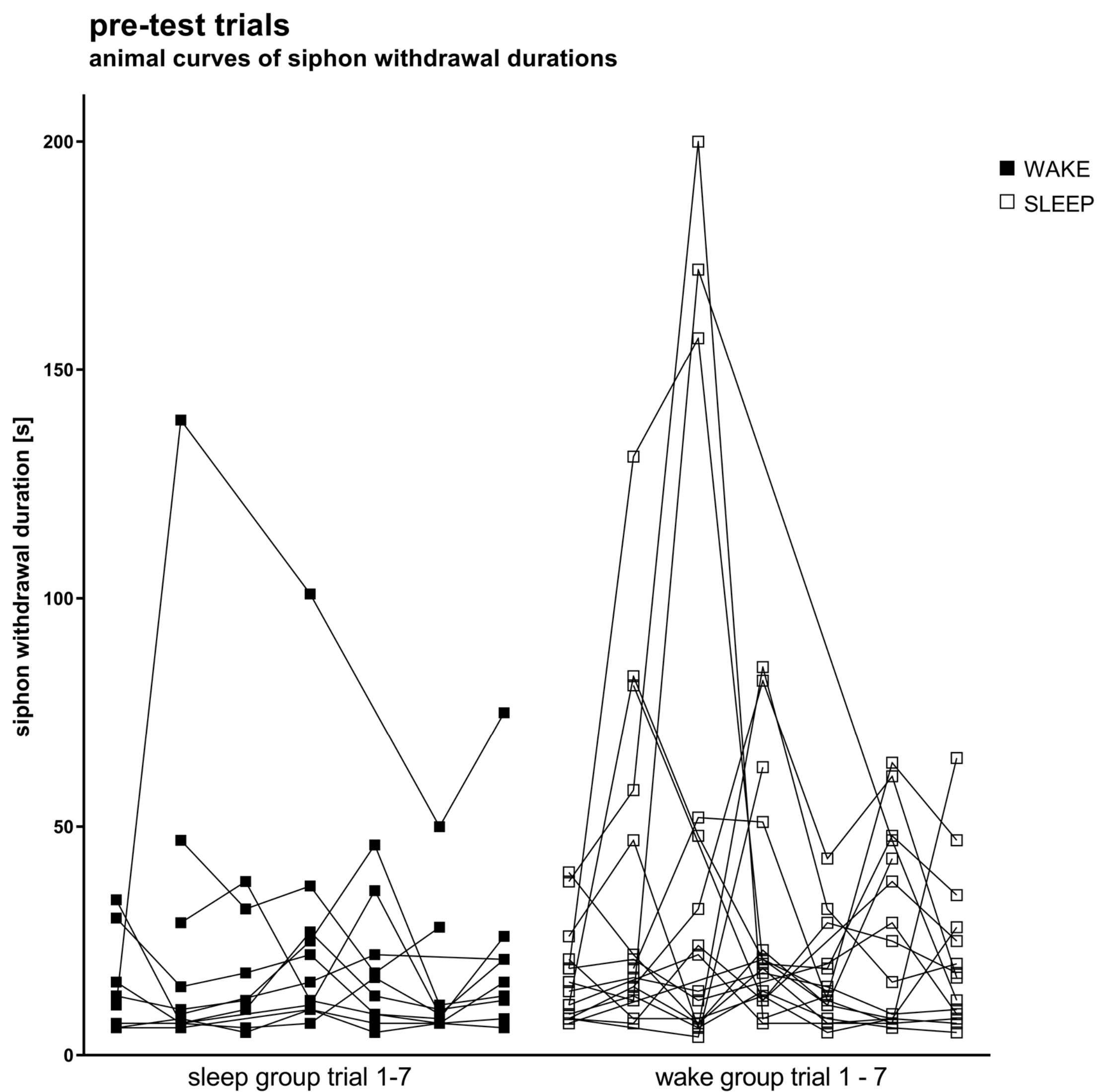

**Supplemental figure 5: Siphon withdrawal durations at three testing points and conditioning.**

Each line represents one experimental animal. Following the pre-test, animals were trained to associate a tactile stimulus of the siphon with an electric shock. 12 animals were sleep deprived thereafter for 6 hours (WAKE), the others served as controls (SLEEP). After the 24h-test, extinction trials of only tactile stimulations were performed. Each test consisted of 7 trials, of only tactile stimulations. The median of the measured siphon withdrawal durations was used as the test result for the individual animal.

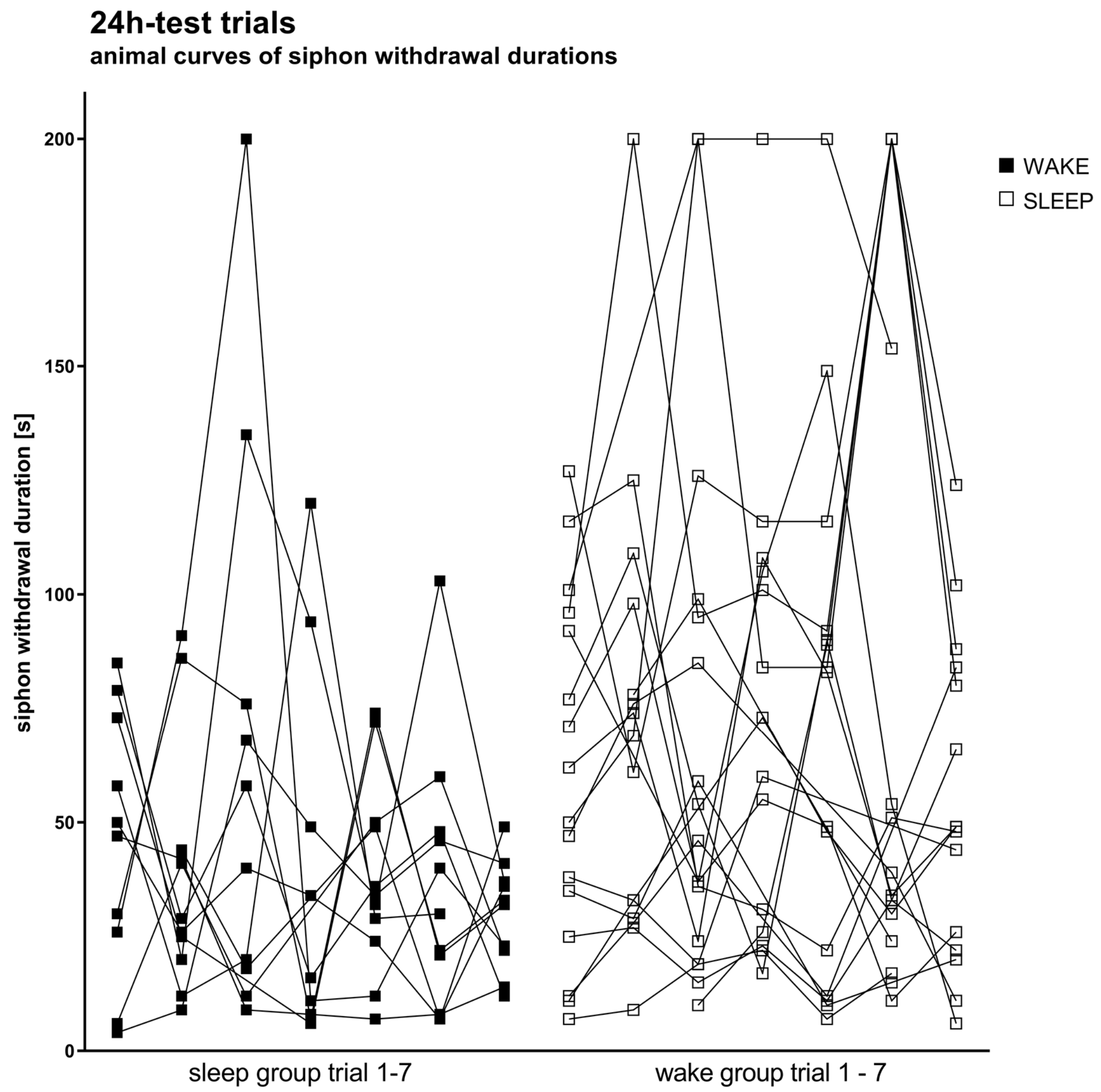
### 3.4.2.3. Single values of withdrawal durations in all tests

To see the individual siphon withdrawal over the course of 7 test trials or 20 training trials, the individual durations are ordered by trial number. There were no dynamics found as a gradual decrease or increase in siphon withdrawal durations after several applications of the conditioned stimulus. Each experimental animal is represented in a curve.

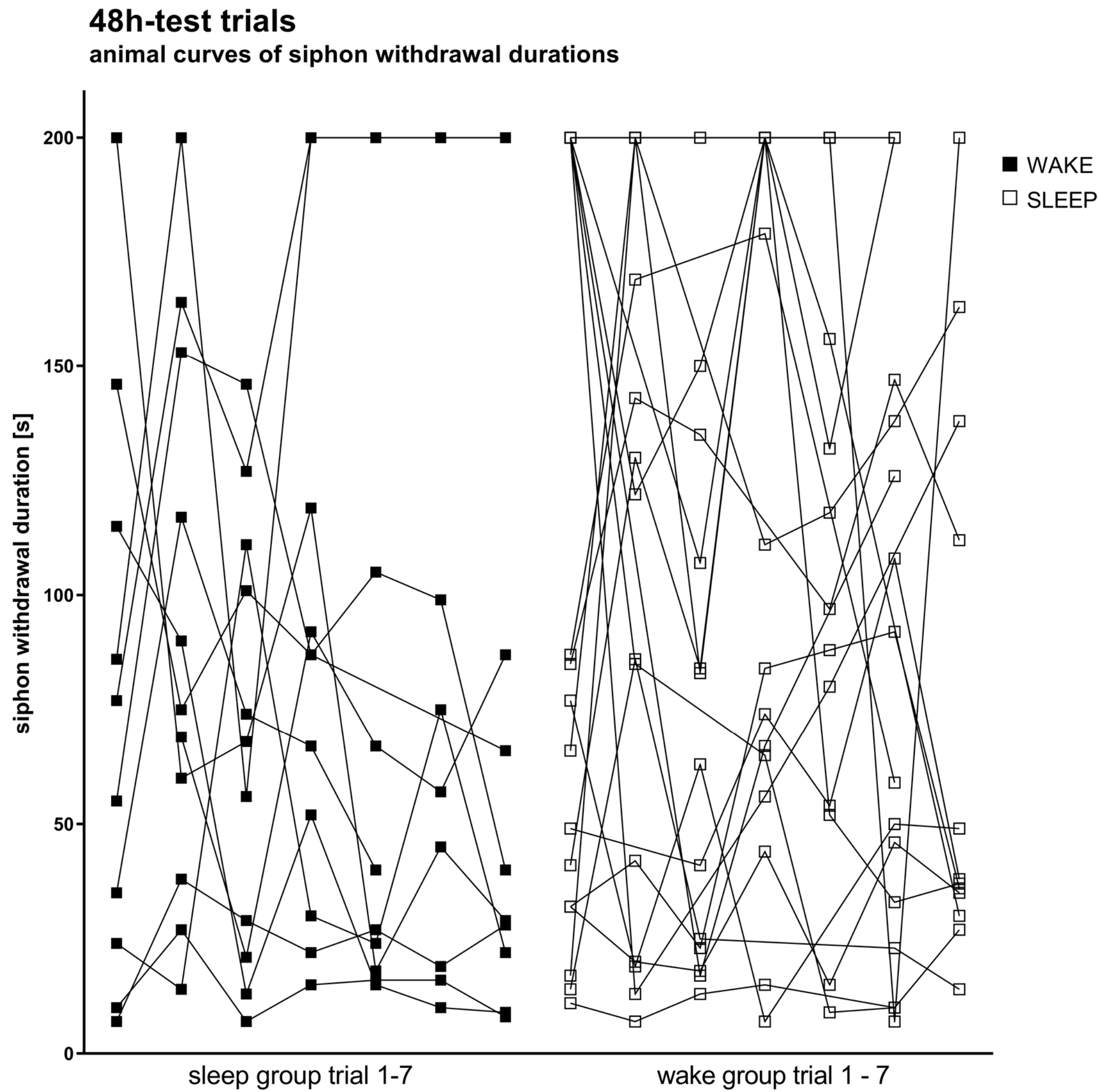
**[A]**



**[B]**



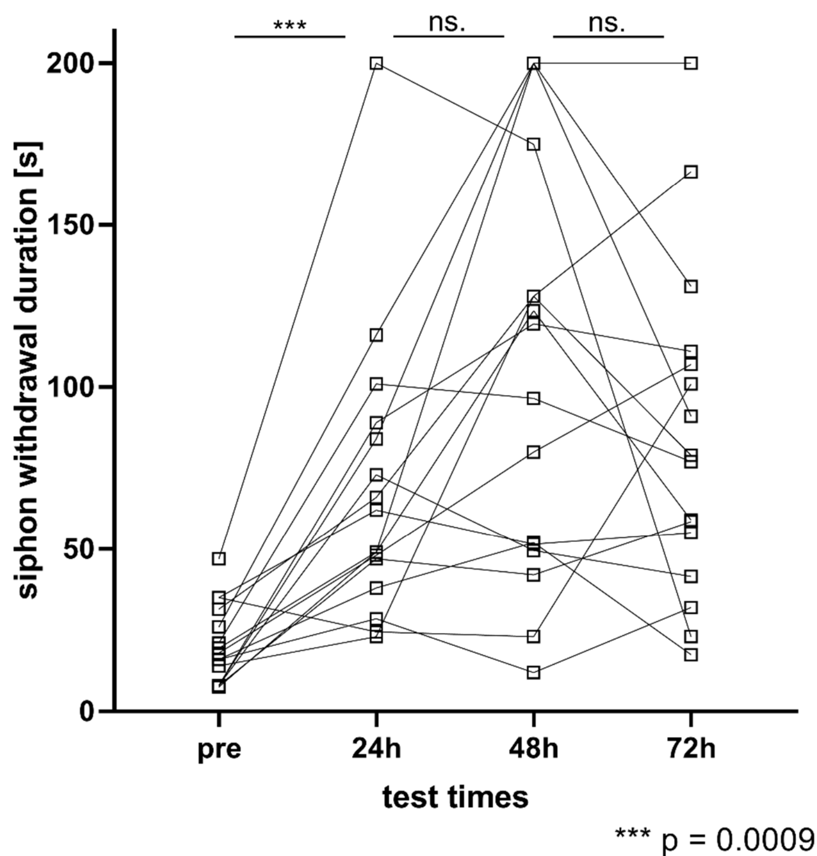
[C]




**Supplemental figure 6: Siphon withdrawal durations ordered by trial in pre-, 24h- and 48h-test ([A], [B] and [C] respectively) in *Aplysia*.**
Each line represents one experimental animal. Siphon withdrawal of 180 s or longer were counted as 200 s, as 180 s was the longest measurable timespan. In SLEEP group, n = 12; n = 18 in WAKE group. In statistical analysis, the median of all available values was taken as the test result of the individual animal.

### 3.4.2.4. Follow up at 72 h of individual siphon withdrawal durations

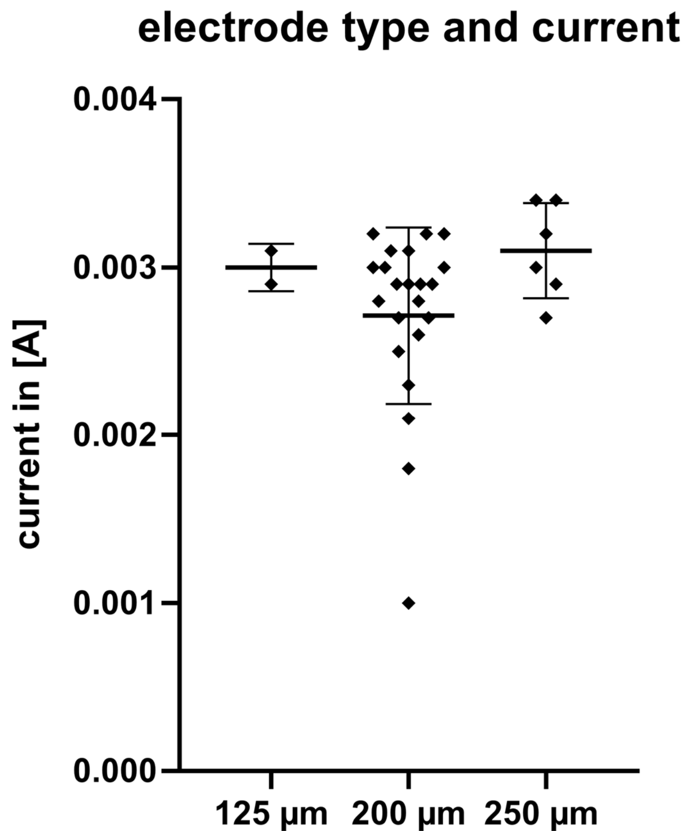
Persistence of conditioning memory was re-assessed 72 hours after acquisition in 16 WAKE group animals. In the mean, siphon withdrawal durations were comparable to 24h- and 48h-test scores. Supplemental figure 7 shows the individual animal's withdrawal durations in all performed tests.




**Supplemental figure 7: Siphon withdrawal duration before and 24 hours after classical conditioning and 24 and 48 hours after extinction training.**
The effect of extinction training was assessed a second time in 16 WAKE group animals. There was no significant change in withdrawal durations between the 48h-test and the 72h-test. Values are the median of 7 test trials, each line represents one experimental animal.

### 3.4.2.5. Implanted wire and resulting current

To proof, that electrode diameter had no impact of the current flow at the application of the unconditioned stimulus, the measured current flows were compared for the three types of electrodes.

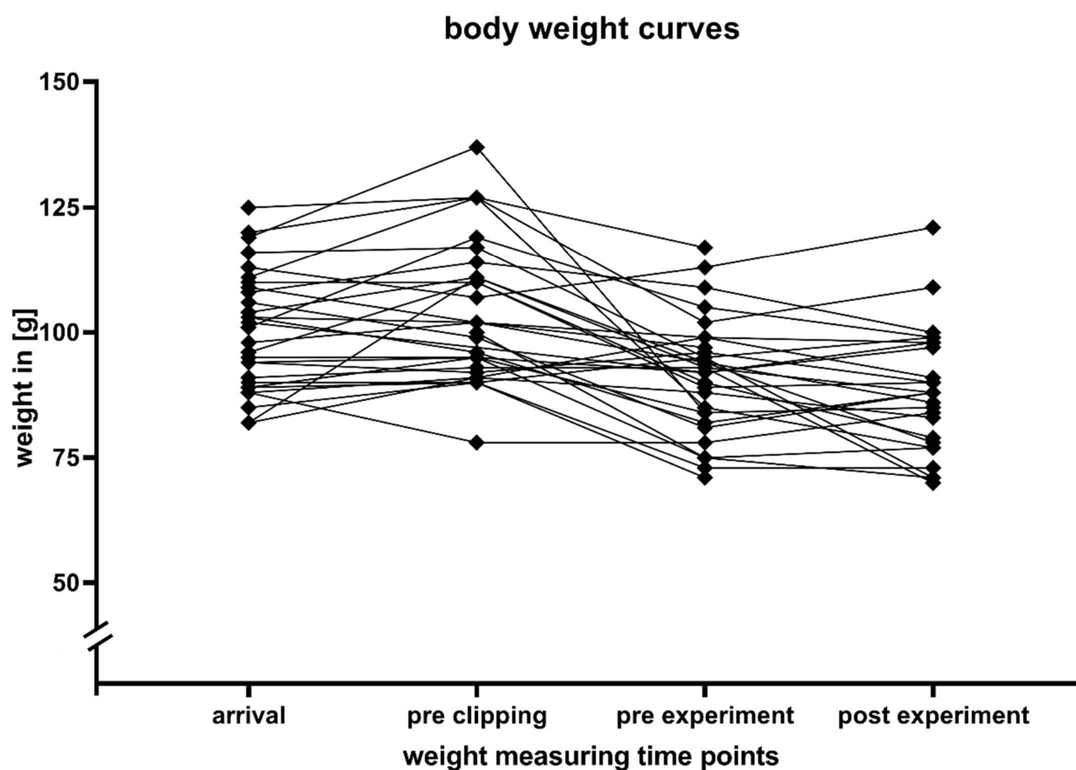



**Supplemental figure 8: Diameters of the electrodes for shock application and resulting current.**
Comparison of the diameters of the used electrodes and resulting current. Three types of electrodes were used in the experiment. To explore possible confounds arising from this fact, the electrode diameter was correlated to the resulting current. There is no significant correlation between the electrode diameter and the resulting current (Pearson's $r = 0.1386$, $p = 0.4652$). Data are pooled for all animals that participated in the experiment, including those that were not considered in the analysis ($n = 30$).

## 3.4.3. Physical condition of experimental animals

### 3.4.3.1. Body weight curves

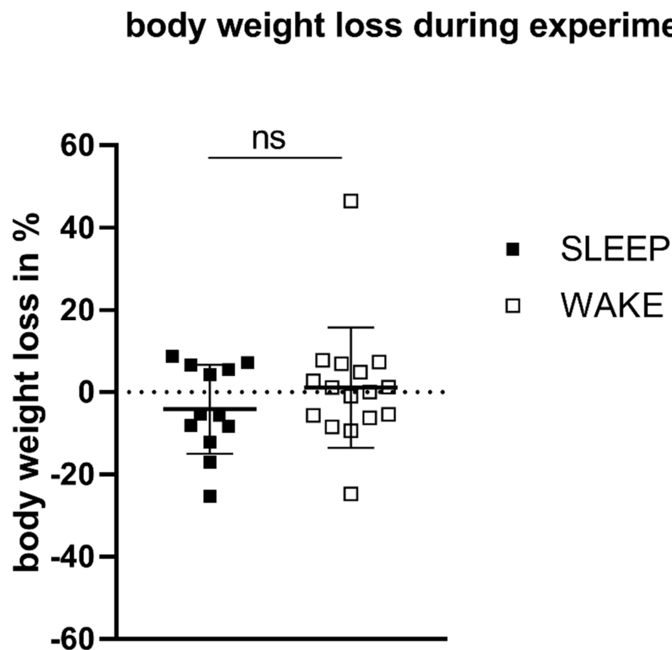
The weight of the experimental animals was monitored regularly to control well-being of the snails. Supplemental figure 9 shows the individual body weight in the progress of the preparations and experiment.




**Supplemental figure 9: Body weight of *Aplysia* in classical conditioning experiment.**
The body weight of all snails was assessed at 4 time points: upon arrival, before parapodectomy, before animals were placed into the experimental boxes and when they were taken back to the home tank. Data are pooled for all animals that were part of the main experiment (n = 30), including those that were not considered in the analysis.

### 3.4.3.2. Weight loss during experiment

Body weight after the end of the experiment was subtracted from the weight before animals were placed into the experimental boxes to assess weight loss during the experiment. There was no significant loss of body weight detectable.




**Supplemental figure 10: Weight loss of SLEEP and WAKE group animals in the experiment.**
The percentual body weight loss from first assessment at arrival to the end of the experiment is depicted for individual *Aplysia*. Data are pooled for all animals that participated in the experiment, including those that were not considered in the analysis (Two data points are missing from measuring after the end of the experiment, n = 28). The bars indicate mean and standard deviation. SLEEP group: mean = - 4.2 %, SD ± 10.8 % (n = 12); WAKE group: mean = + 1.1 %, SD ± 14.6 % (n = 16).

# 4. Discussion

In recent years, several publications focused on how operant conditioning memory in *Aplysia californica* is influenced by sleep (Krishnan et al. 2016a, Krishnan et al. 2016b, Vorster and Born 2017, Vorster and Born 2018). Sleep deprivation impaired memory consolidation but interestingly had a beneficial effect on the extinction of operant memory. In this work it was tested, whether an even simpler type of learning, namely classical conditioning, relies on sleep for consolidation. There are four main findings:

1. All classically conditioned animals showed a clear learning effect in a memory test after 24 hours.
2. The induced memory was very robust, as it persisted for at least 72 hours
3. Consolidation was not influenced by sleep deprivation of 6 hours.
4. An extinction training did neither show an immediate nor a long-term effect.

## 4.1. Classical conditioning and sleep

### 4.1.1. Conditioning was effective

The siphon withdrawal reflex of classically conditioned *Aplysia* was significantly prolonged after a 24-hour consolidation and retention interval, when compared to the naïve reflex duration. Therefore, the performed training was effective. In follow-up tests after 48 and 72 hours, siphon withdrawal durations were still elevated in comparison to before training. This persistence revealed the strength of the learned association between the conditioned and unconditioned stimulus.

This first finding stands in line with the original publication of classical conditioning of the siphon withdrawal reflex and several later experiments that further explored the reflex (Carew et al. 1981b, Carew, Walters and Kandel 1981a, Carew et al. 1983, Hawkins et al. 1986, Hawkins et al. 1989, Hawkins, Greene and Kandel 1998). That conditioning memory was existent for more than 72 hours, was previously shown by Carew et al. (1981b).

### 4.1.2. Classical conditioning was not impaired by sleep deprivation

It was now asked, whether sleep deprivation of 6 hours following training, would impair consolidation of the prolonged siphon withdrawal reflex. If so, in the retrieval test 24 hours after training, a difference could be seen between sleep deprived and control animals. As the results of both groups are not significantly different, it can be postulated that sleep deprivation of 6 hours did not affect consolidation of classical conditioning of the siphon withdrawal reflex. In favour of this finding, no significant correlation was found between the sleep duration in 6 and 24 hours after training and the performance in the 24h-test. In case sleep benefits conditioning, animals with longer sleep durations would be expected to exhibit longer withdrawal durations. This was not the case.

These results are consistent with those of Hussaini et al. (2009), who showed that in honeybees, memory of classical odour conditioning did not differ after a period of sleep or sleep deprivation. In that study, one group of bees was sleep deprived for 16 hours directly after training. Yet, conditioning memory was intact at the retrieval test after 24 hours. This indicates that consolidation of classical conditioning memory is independent of sleep.

In rodents, the situation is rather ambiguous. Classical aversive conditioning and sleep deprivation in rodents was performed by Graves et al. (2003), Cai et al. (2009) and Kumar and Jha (2012). Foot shocks were paired to sound signals, a paradigm called cued fear conditioning. Graves et al. (2003) deprived mice from sleep for 5 h following training after a single trial of cued and context fear conditioning. Sleep deprivation caused disturbance to consolidation of context fear conditioning, while consolidation of cued fear conditioning was not impaired.

In contrast, Cai et al. (2009) found impaired cued fear memory by sleep deprivation that was performed directly after training with a duration of 12 hours. They criticized Graves et al. for training and testing the experimental animals during their natural sleeping phase. Moreover, Cai et al. showed, that gentle handling itself causes disturbance to cued fear memory, a method that had been used to sleep deprive the mice in Graves et al. The latter group had not controlled for not sleep-related effects of mere handling. However, both groups share a confound they did not consider: Mice were classically and context conditioned in

one context but the cued fear conditioning was tested in a different context. This change required for a memory transfer to the new context. It is possible, that sleep deprivation impaired the animals' ability to perform such a transfer, rather than interfering with consolidation.

Kumar and Jha (2012) also found diminished learning, when sleep depriving rats for 6 hours directly after 10 trial conditioning training. However, this finding is to be questioned, as the mice were trained at 7:00 pm, i.e. the beginning of their active phase. As sleep deprivation was performed consequently to training, this was still within the phase of natural wakefulness. Unfortunately, it was not shown, whether sleep deprivation actually resulted in less sleep in the deprivation group. Therefore, it remains unproven, whether differences in the amount of sleep account for diverging learning outcomes.

In summary, data in rodents are not conclusive as to whether or not consolidation of cued fear memory is sleep dependent.

It appears that in *Drosophila*, as opposed to *Aplysia* and honeybees, classical conditioning is influenced by sleep deprivation in some cases. Le Glou et al. (2012) studied the effect of 4 h-sleep deprivation on long-term memory of classical conditioning. Flies were trained to associate one odour with an electric shock, another odour served as control. Memory was assessed by the choice of direction in a T-maze, that contained one of the odours in each arm. It was found, that sleep deprivation performed between 4 to 16 hours after conditioning, impaired classical conditioning memory consolidation. Li, Yu and Guo (2009) further showed that sleep deprivation also impaired subsequent formation of short-term memory, in the same classical olfactory conditioning.

Yet, this conclusion is undermined by a methodological shortcoming: the memory test using the T-maze is an indirect assessment of conditioning memory. Like in the studies in mice, this test design requires a memory transfer to a new context from acquisition to memory test. Therefore, the memory test assessed more qualities of memory than only olfactory conditioning. These additional memory dimensions, like spatial information, might have been sleep-dependent and thus account for the impaired memory after sleep deprivation.

Another aspect that might account for the divergent effects in the examples described above, could be differences in the underlying neuronal circuits. In mammals, fear conditioning involves the amygdala (Paré, Quirk and Ledoux 2004), a structure that plays a major role in emotional responses. *Drosophila* and honeybees, despite being invertebrates, possess highly organized brains. Yet the number of neurons in the brain of honeybees, is about 3 - 10 times that of *Drosophila*. It can process complex information like locations, encoded in the waggle dance. In both species, the so-called mushroom bodies are involved in aversive classical conditioning (Davis 2015, Tedjakumala and Giurfa 2013). The mushroom bodies process complex information of the insect's internal state and appetitive or aversive external stimuli. Further, they serve functions in memory and learning. Different populations of neurons within the mushroom bodies serve specific purposes (Stopfer 2014). In contrast, the neuronal circuit of the siphon withdrawal reflex of *Aplysia* is simple. It involves a sensory-motor synapse and a countable number of modifying excitatory and inhibitory interneurons. In conditioning, these interneurons receive input from sensory neurons of the tail.

However, the complexity of the involved brain structures, does not indicate sleep dependency of consolidation. Comparison the described studies in mammals reveals, that the conclusions on sleep dependency of classical conditioning are divergent. Also, in *Drosophila* and honeybees, who possess similarly structured brains, the findings are incoherent.

Summarizing the most convincing studies of the above mentioned, data points into the direction, that classical conditioning as a very simple type of learning is consolidated independently of sleep.

### 4.1.3. Sleep levelled learning

While sleep deprivation in this study did not interfere with consolidation of classical conditioning, it seems that sleep did elicit an effect. In comparison to the WAKE group, the variance of performance in the 24h-test was significantly smaller in the SLEEP group. This finding replicates observations of Vorster and Born (2017) and Vorster and Born (2018). A possible interpretation might be, that sleep provides optimized conditions for consolidation, resulting in stable results in a group of experimental subjects. Indeed, the synaptic homoeostasis

hypothesis by Tononi and Cirelli (2003) provides the theoretical framework to explain this outcome. They argue that endless potentiation of synapses during wakefulness and learning would result in a collapse of the system by insatiable consumption of energy, cellular supplies and space. General synaptic down-scaling during sleep thus ensures functioning of the neuronal network. During training, a stable input-output association between conditioned stimulus and prolonged siphon withdrawal was established. According to the hypothesis, all synapses were down-scaled in the sleeping subjects, but those synapses that were potentiated through learning to a fewer extend. Through synaptic renormalization, the signal strength of the reflex thus would be levelled and stabilized. This individual effect could then be measured in less varying learning outcomes in the sleep group.

## 4.2. Extinction

In the present work, an extinction training was performed 24 hours after conditioning, but no immediate or long-term effect was found. During the presentation of 7 tactile siphon stimulations, no decline in withdrawal duration could be seen. Thereafter, another 26 tactile stimulations were applied. In a memory test 48 hours after conditioning, it was controlled if extinction had set in with a delay. But rather than shortened, siphon withdrawal durations were slightly longer. A follow-up test revealed that the prolongation in siphon withdrawal persisted at least 72 hours after conditioning.

Extinction of the conditioned prolongation in siphon withdrawal, was first described by Carew et al. (1981b). There, the extinction training was performed directly after conditioning and consisted in tactile siphon stimulations, without paired tail shocks. The group reported a successive decline in siphon withdrawal duration within 10 trials. A gradual decline of the conditioned response, is a characteristic feature of extinction learning (Zyuzina and Balaban 2016).

Why was there no evidence for extinction of classical conditioning of the siphon withdrawal reflex in this work? There are several possible explanations:

**1. Conditioning memory was too strong to be extinguished.**

Contrary to this study, Carew et al. (1981b) performed extinction training directly after acquisition. They did not perform a retrieval test for long-term memory e.g. after 24 hours. Unfortunately, there are no further studies examining extinction of this paradigm. As there was a 24-hour break between training and retrieval test, memory could consolidate, as opposed to the experiment of Crew et al. Possibly, consolidation made the conditioning memory more resistant to extinction, see Thiede et al. (2021).

However, in other classical conditioning paradigms in *Aplysia*, extinction training 24 hours after acquisition was successful. Colwill, Absher and Roberts (1988) trained *Aplysia* for 8 days to associate a context with electric shocks. This connection was extinguished through several unreinforced context expositions on two consecutive days. In an appetitive conditioning protocol, performed by Colwill et al. (1997), the association of a taste and consequent food reward was also successfully extinguished one day after acquisition. These examples show, that it is possible to extinguish classically conditioned memory, after a 24-hour consolidation period, in *Aplysia*. Recently, Vorster and Born (2018) showed extinction of an operant conditioning memory, that had been acquired 24 hours before. The extinction memory was still preserved another 24 hours later, i.e. 48 hours after acquisition.

To further explore extinction of classical conditioning of the siphon withdrawal reflex, one might have to perform an even longer extinction training. Following the example of Colwill et al.(1988), it could be conducted on two subsequent days. Extinction was found to be strongly linked to context changes in many species (Borquez et al. 2017, Bouton and Moody 2004). It would be interesting to investigate, whether context changes could support extinction also in *Aplysia*. Another approach to achieve extinction could be, to weaken the association between tactile stimulus and shock, by modifying the protocol. Like in Hawkins et al. (1986), 20 trials were applied here, with a break of 45 min after 10 trials. Spaced training was shown to induce stronger memory (Carew et al. (1972); Menzel, Manz and Greggers (2001); Litman and Davachi (2008)). Applying for

instance only 10 trials in one training block, might reduce the resistance of conditioning memory against extinction.

**2. The applied tactile stimulus was too strong and provoked its own sensitization or reinforcement**

It could be that the tactile stimulus itself lead to a sensitization in the *Aplysia*. Consequently, in any of the memory tests, one would see prolonged siphon withdrawal as an expression of this sensitization. Further, in the extinction training, a strong tactile stimulus would additionally reinforce the sensitized reaction, rather than extinguishing it. This would explain why in the memory test after extinction training, siphon withdrawal durations persisted to be prolonged.

Yet, this seems rather unlikely, as the conditioned stimulus itself did not lead to any change in behaviour when it was applied solely in the pre-test. Here, the tactile stimulus was applied 7 times, to test the animal's naïve siphon withdrawal duration. If the tactile stimulus was of relevance to the animal, one would await the withdrawal duration within the 7 trials to either increase (as in sensitization), or decrease (as in habituation) (Carew et al. 1972, Pinsker et al. 1970). As this was not seen, the conditioned stimulus does not appear a plausible cause for prolonged siphon withdrawal in the retention tests.

**3. Extinction training served as a reminder learning and strengthened the original memory rather than resulting in extinction.**

This explanation follows the "internal reinforcement theory" by Eisenhardt and Menzel (2007). It aims at explaining, why in some cases extinction training does not result in reversal of the originally acquired memory. The basic principle of this theory resembles second-order conditioning. There, a conditioned stimulus can serve as an unconditioned stimulus for a second conditioned stimulus. That second-order conditioning is possible in *Aplysia* has been shown by Hawkins et al. (1998). There, a conditioned stimulus (CS1, tactile stimulus to one side of the siphon) was paired to an electric shock (the unconditioned stimulus, US) provoking prolonged siphon withdrawal. The longer siphon withdrawal was the "unconditioned response" (UR). In a second conditioning phase, a different

conditioned stimulus (CS2, tactile stimulation of the other side of the siphon) was paired to the CS1, but no shock followed. The observed reaction to the CS2 in a retrieval test was prolonged siphon withdrawal (the UR), even though the CS2 was never paired to a shock. Animals had successfully learned the connection of CS1 and US, as the shock (the US) was not necessary to elicit prolonged siphon withdrawal (the UR). In other words, the CS1 had become the US for the CS2.

The idea underlying the internal reinforcement theory is, that the tactile siphon stimulation during extinction, serves as the CS1 and CS2 in one. Instead of a reversal learning as during extinction, therefore a reminder training happens. In that way, even though the electric shock, as the external reinforcement is not presented during extinction training, every CS presentation will be followed by the unconditioned response (UR). Thereby, the connection between CS1/2 and UR will be strengthened, in the words of Eisenhardt et al. it will be “internally reinforced”. This is opposing extinction training, where the learned unconditioned response to the CS will gradually fade.

If the internal consolidation theory applies to the present experiment, the conducted extinction training served as a reminder training. Consequently, neither could there be seen a reduction in siphon withdrawal during extinction, nor 24 hours afterwards, as rather than being extinguished, conditioning memory would have been reinforced.

Eisenhardt et al. suggested, that with an increasing number of extinction trials, the memory of reinforcement would become weaker and thus the extinction effect stronger. In the extinction training of this work, a high number of trials was applied (33 extinction trials as opposed to 20 acquisition trials). One might thus suppose, that the extinguishing effect should be predominant over a possible reinforcement. Yet, it is to be mentioned, that the frequency of trials in extinction training was doubled after the 7$^{th}$ trial. This might have added to a reinforcement, if 2.5 min was not enough time for the *Aplysia* to fully relax. Then during the whole extinction training, a state of excitation in the sense of almost constant withdrawal, might have been established. Every further tactile stimulation would then strengthen the association between CS1/2 and the unconditioned response (= prolonged siphon withdrawal). In summary, the internal reinforcement theory

would be a possible explanation, why extinction training was not effective. To finally define the resulting effect of extinction training, it might be useful to conduct extinction on 2 consecutive days with longer inter trial intervals.

**4. There was a stronger non-associative effect (sensitization), that masked the extinction of conditioning.**

As shown by Carew et al. (1981b) and Carew et al. (1983), classical conditioning of the siphon withdrawal reflex always has a non-associative component. Carew et al. (1981b) demonstrated, that paired training of tactile stimulation and shock produced longer siphon withdrawal, than training with shock only i.e. sensitization. This difference was only observed in a memory test after 24 hours. None of the groups showed increasing siphon withdrawal durations during training. They wondered, why the sensitization effect did not occur already during training, like in earlier sensitization studies. Carew, Castellucci and Kandel (1979) relate sensitization after only 1 trial, when tested after 2 hours.

In control experiments, Carew et al. (1981b) found, that sensitization only became visible after at least 30 min and began to wear off after the second day post training. Siphon withdrawal durations in classically conditioned *Aplysia* were longer than in sensitized animals, when tested 24 and 48 hours after training. Moreover, conditioning memory persisted for 4 days, sensitization memory for only 2 days. No further studies could be found, which relate persistence of either types of learning. Likewise, our data indicate, that the memory of paired training persisted for at least 3 days. Two years after their original publication, Carew et al. (1983) again directly compared conditioning training to sensitization training. They could confirm, that conditioning produced significantly longer siphon withdrawal durations.

It might be, that with increasing impact of the unconditioned stimulus, the sensitizing effect exceeds the conditioning effect. Two findings point into that direction: Carew et al. (1981b) as well as Pinsker et al. (1973) found, that a higher number of sensitization trials lead to longer siphon withdrawal. Hawkins et al. (1989) directly compared the effects of different strength shocks: They conditioned two groups of *Aplysia*, with paired presentation of a conditioned

stimulus an electric shock of either 40 or 60 mA intensity. A second conditioned stimulus was applied without subsequent shock, i.e. unpaired. In animals trained with 40 mA, siphon withdrawal after 24 hours was longer in response to the conditioned stimulus, which had been paired with the shock. The group trained with a 60 mA shock, did not show a difference between the reactions to the paired or unpaired conditioned stimulus in the 24h-test. The stronger shock thus eliminated the differential effect of paired and unpaired training and animals were primarily sensitized.

Interestingly, in the 60 mA group, a differential effect became visible after 48 hours: animals now showed longer siphon withdrawal to the conditioned stimulus that had been followed by the shock. This indicates, that there was a conditioning learning, that had been masked by the non-associative effect in the 24h-test.

It could be that in the main experiment of this work, the non-associative surpassed the associative component and thus the test results reflect strength of sensitization instead of conditioning. This would explain why in extinction training, there was neither a gradual reduction of the withdrawal durations, nor a long-term effect 24 hours later: a long-term sensitized *Aplysia* would keep reacting to even to weak tactile stimulation with prolonged siphon withdrawal durations.

In this work, much less current was used to train *Aplysia*, yet animals reacted strongly to the shocks. This might be attributed to the use of implanted electrodes and the fact that, depending on the place of origin of *Aplysia*, withdrawal durations can vary (Fischer et al. 2000). Comparability is complicated by the incomplete descriptions of former experiments of classical conditioning of the siphon withdrawal reflex. However, judging by behavioural criteria like inking to shock and group mean withdrawal duration, reactions to the electric shock were comparable to previous studies. It remains unclear, if the current strength of former publications, had the same physiological impact to *Aplysia* as in this experiment.

In conclusion, the lack of a decrease of siphon withdrawal durations during extinction training and 24 hours thereafter, could be interpreted as a sign of sensitization. Considering, that sensitization is always present in conditioning of the siphon withdrawal reflex, this seems to be the most likely explanation

(compare Thiede et al. (2021)). To finally solve this issue, a control group for sensitization (i.e. US only group) would be needed. Moreover, reducing the shock intensity might weaken the non-associative effect of training. Using a weaker shock, bears the risk of not seeing conditioning at all, which was the reason to decide on an intermediate shock intensity of 7 V / 2.8 mA.

## 4.3. Study design

### 4.3.1. Low unconditioned stimulus intensity

For unknown reasons, currents that provoked defensive behaviour in the present work were much smaller than in any of the former publications. In accordance with Carew et al. (1981b), Carew et al. (1983) and Hawkins et al. (1986), in this experiment, the electrodes used for shock application were implanted, to hold the strength of the stimulus constant throughout repeated applications. Other groups held electrodes manually onto or close to the skin of the snails (Walters et al. 1979, Walters, Carew and Kandel 1981, Hawkins et al. 1986, Colwill et al. 1988, Hawkins et al. 1989). However, information about the applied current and the setup of the electric circuit in these cases are incomplete. Therefore, it proved to be harder than expected, to establish a setup that would produce reliable conditioning which could then be extinguished.

Independently of whether handheld or implanted electrodes had been used, former authors applied higher levels in voltage and current (see also Thiede et al. (2021)). In the present work, applied voltages were of 4.5 to 10 V, with currents of 1.0 – 5.7 mA as a tail shock (US). Some authors applied weak electric shocks as the conditioned stimulus, instead of tactile stimulation of the siphon. The settings they chose as weak current for the conditioned stimulus, were within the range of what was used as the unconditioned stimulus here. For the unconditioned stimulus in turn, these groups used shocks of at least 10-fold the weak current, both with externally hand-held and implanted electrodes. For example, Carew et al. (1983), who also implanted a pair of electrodes into the tail, applicated current within the range of 20 – 30 mA, with an in series resistor

of 10 kΩ. When electrodes were held into the water close to the animal, up to 75 mA were applied (Hawkins et al. 1986).

The aversive effect of an electric shock depends in great part on the flow of current (Fish and Geddes 2009). But even though current flow in this work was much weaker, siphon withdrawal durations were comparable to those of older papers. Also, like in former publications, animals in this work reacted with ink and opaline release. As the snails showed defensive behaviour, the weaker shocks used here, were clearly identified as noxious.

Cohorts of *Aplysia*, collected from different places and during different times of the year, tend to show varying characteristics in thresholds for reflexive behaviour (Fischer et al. 2000). That fewer current was sufficient to elicit inking, could therefore be just a feature of a particular cohort. But as the setup was developed and evaluated within 12 months, using multiple animal deliveries, this explanation seems rather unlikely. Rather, it could be questioned, if the given specifications of shock applications in earlier studies are correct. It might be, that only a small part of the electrical current had reached the animals and that the majority of the electricity was carried by the water. The actual current flow in the animal tissue was thus seemingly not well controlled and probably varying between the individual *Aplysia* and between different trials in one snail. In contrast, the setup described here, allows for precise adjustment of the shock intensity and guarantees equal preconditions for every experimental animal.

### 4.3.2. Duration of sleep deprivation and memory effect

The main experiment of this work was conducted with a sleep versus sleep deprivation group. The two groups did not differ in their learning outcome after 24 hours. It could be argued that 6 hours of sleep deprivation were insufficient to interfere with memory consolidation.

An established method to deprive animals from sleep (Vorster and Born 2018, Vorster and Born 2017) was adopted. By means of a flat, flexible plastic ruler, snails were removed from the surface they stuck to, if they showed no movement for more than one minute. As floating provokes reflexive righting, *Aplysia* will

wake up. This method was adapted and refined to reduce the possible interference with classical conditioning of the siphon withdrawal reflex.

*Aplysia* were sleep deprived directly after classical conditioning for 6 hours. Training ended shortly before the beginning of the dark phase; sleep deprivation thus lay within the first half of the inactive period of *Aplysia californica*. In former publications, the length of conducted sleep deprivation in different species varies greatly from 80 min (Borquez et al. 2014) to 72 h (Pinheiro-da-Silva et al. 2017). Previous experiments in *Aplysia* performed sleep deprivation of 9 hours (Krishnan et al. 2016a) and 17 hours (Vorster and Born 2018, Vorster and Born 2017). In classically conditioned mice, Graves et al. (2003) found not duration, but timing of sleep deprivation relevant for whether or not it had an effect on consolidation. The sensitive period lay within 5 hours after training. Such time windows, critical for sleep deprivation to impair consolidation, were found in various paradigms and lay between 4 and 6 hours after training (Fu et al. 2007, Graves et al. 2003, Hagewoud et al. 2010, Palchykova et al. 2006a, Palchykova et al. 2006b, Prince et al. 2014). In classical conditioning of invertebrates, only in fruit flies a memory effect of sleep deprivation was found. Here the duration of sleep deprivation was just 4 hours (Le Glou et al. 2012). In view of these examples, it can be argued that sleep deprivation of 6 hours starting directly after training, should be sufficient to interfere with consolidation of classical conditioning in *Aplysia*, if the memory type is susceptible to sleep deprivation.

### 4.3.3. Homoeostatic impact and stress induction of sleep deprivation method

Another reason to sleep deprive *Aplysia* only for 6 hours, was to minimize undesirable homoeostatic effects of sleep loss. Such homoeostatic alterations might diminish performance in the memory test via impaired retrieval. To further reduce the impact of sleep deprivation on performance in the 24h-test, animals were left undisturbed for about 14 hours before testing. During this time span, recovery sleep was permitted. Interestingly snails did not exhibit rebound sleep, neither in the 14 hours after sleep deprivation, nor in the following night. This might indicate that 6 hours of sleep deprivation are of minor physiological

relevance. The influence on performance in the retrieval test could then be considered equally insignificant.

Additionally, sleep depriving the snails only during half of the resting phase, allowed the reduction of necessary handling. The method applied to keep *Aplysia* awake was harmless; simply sliding a flat object between foot and substrate from the anterior to the posterior part of the animal. But as conditioning involved shocks to the side of the tail, any sensory stimulation to the tail afterwards was to be avoided, to prevent interference with the conditioning paradigm.

As suggested by Krishnan et al. (2016a), a feasible control for this confounding factor, could be similar handling of the control group during the active time phase. Rather than this, it was intended to motivate the snails to actively stay awake and thereby reducing the necessary amount of handling to keep the *Aplysia* awake. To this end, animals were presented the odour of food during the sleep deprivation period.

#### 4.3.3.1. Starvation as motivation

To further enhance the animals' motivation, the snails were deprived from food for 5 days prior to training. In preliminary experiments it became evident, that precedent food deprivation combined with presentation of food odour during the intervention night, succeeded in motivating *Aplysia* to stay awake.

In *Drosophila*, Thimgan et al. (2010) and Keene et al. (2010) already used food deprivation as a method of sleep deprivation. Starvation surely affects many, mainly metabolic processes in an organism, but it also leads to a different motivational state. This in turn increases locomotion as demonstrated e.g. in rats (Jacobs and McGinty (1971), Finger (1951), Finger (1965), Danguir and Nicolaidis (1979)), *Drosophila* (Lee and Park (2004), Erion et al. (2012)) and *Aplysia fasciata* (Susswein 1984). In a detailed description of *Aplysia fasciata*'s behaviour following food deprivation, Susswein (1984) reported a decrease of time spent immobile. This state of immobility most probably classifies as sleep, following the criteria of Vorster et al. (2014). Enhancement of the snail's motivational state also positively influences training: Advokat (1980) found, that sated *Aplysia* exhibit suppression of siphon withdrawal and inking.

To ensure all WAKE group animals were sleep deprived, additional manual handling could not be totally spared. Yet, a second conclusion can be drawn from the fact, that learning did not differ in the sleep and sleep deprivation group: the applied sleep deprivation itself did not impair memory consolidation.

## 4.4. Study strengths

Despite the limitations of this study, some unique features can be pointed out, that substantiate its findings: To hold conditions constant for all subjects, relevant environmental parameters were controlled: constant water temperature in home tanks and experimental boxes, continuous aeration, high water quality (with regular checks for nitrate, nitrite, ammoniac, etc) and a constant 12 h/12 h day / night rhythm. Animals' well-being was ensured by regular feeding every second day before and after the experiment, with intake and weight controls at strategic points before, during and after the experiment. Any unusual behaviour was registered, such as egg-laying or inking outside conditioning.

The experimental setup was also designed to provide constant conditions for all experimental animals and to allow for surveillance of experimental parameters. By implanting electrodes for shock application into the animals' tail region, the range of current flow was held extremely small (1.0 – 3.4 mA). Besides, current flow was measured during every shock, to immediately detect any irregularities in the application. The experimental boxes were monitored 24 hours per day, by means of an infrared camera. Automatic sleep scoring was manually controlled and unwanted occurrences during the night-time could have been detected. To allow for blind scoring, animals' reactions were videotaped in all tests and training.

It can thereby be ensured, that experimental animals were of good health and that the experimental procedures did not impair well-being of the *Aplysia*. The siphon withdrawal reflex as part of a defensive mechanism in *Aplysia*, is subject to change by various influential factors. It is therefore imperative to guarantee optimal conditions and to continuously control animals' heath state, to minimize

possible sources of error. Even more so, as group sizes tend to be fairly small in *Aplysia* experiments.

No other publication could be found, which states siphon withdrawal durations during acquisition. Responses during conditioning were found here, to be a parameter, which is important to be monitored. *Aplysia* in this experiment exhibited levelled pre-test scores, but reactions during acquisition were significantly different for the two experimental groups. As external parameters were held constant, these differences can only be explained by unknown internal reasons. The data points into the direction, that longer siphon withdrawal in conditioning, might be associated with longer siphon withdrawal after 24 and 48 hours (see supplemental figures 9 and 10). As such a correlation would bias the results, both pre-test and conditioning siphon withdrawal durations should be measured. Eventual differences might then be equalled out, either by balanced assignment of the individual animals to the experimental groups or by post-hoc statistical compensation. In older publications, intervention and control group were often balanced according to pre-test siphon withdrawal duration. Individual differences in withdrawal reaction to electric shocks, might be a confounding factor, that was previously not registered and thus not compensated for.

## 4.5. Conclusion

In this study, the siphon withdrawal reflex of *Aplysia californica* was classically conditioned, based on the work of Carew et al. (1981b). Equal to previous publications, training resulted in prolonged reflex durations, which persisted for at least 72 hours. However, the change in behaviour could not clearly be attributed to conditioning. It had been reported before, that conditioning of the siphon withdrawal reflex always comprises sensitization (Carew et al. 1983). In summary, training in this study resulted most probably in combined non-associative and associative learning. In any case, it was a mechanistically rather simple form of learning, and its consolidation was not depending on sleep. The performed extinction training was not sufficient in reversing the prolongation of siphon withdrawal durations. Hypothetically, this can be taken as evidence for a predominant sensitisation component in the learned behaviour.

In a comprehensive article, Vorster and Born (2015) compare sleep effects on different types of learning in mammalian and non-mammalian species. In mammals, only learning that involves the hippocampus was found to critically depend on sleep. Simple classical conditioning in turn was not influenced by sleep. In invertebrates, operant conditioning or inhibitory learning, but not classical conditioning, were found to be sleep dependent. The present study is further backing the hypothesis, that sleep effects are only found in learning of higher complexity than classical conditioning, compare Thiede et al. (2021). The results of this work can now be contrasted with the study of Vorster and Born (2017), who showed that sleep benefits inhibitory operant conditioning in *Aplysia*.

We already know, that there is a link between sleep and learning. Yet we lack complete understanding of the cellular mechanisms that underly the findings in imaging and on the behavioural level. It was hypothesised, that memory is consolidated during sleep by redistribution of learned content (active system consolidation theory, Born and Wilhelm (2012)). In the monosynaptic siphon withdrawal reflex, long-term prolonged withdrawal is caused by long-term potentiation of the sensory-motor synapse (Glanzman 2013). In accordance with the active system consolidation theory, no sleep effect would be expected in

classical conditioning of the siphon withdrawal reflex, as it represents local enhancement without engram displacement.

It would be tempting to explore, whether subsequent modifications of classical conditioning memory, like extinction learning and second-order conditioning depend on sleep to be consolidated. Similarly, other simple non-associative learning like habituation could be scrutinized in that respect. The setup provided here, serves as useful groundwork, to extensively study interdependencies of basic learning and sleep on a behavioural level. Comparable behavioural data will allow future exploration of cellular and electrophysiological correlates of sleep effects on learning and memory.

# 5. Summary

Sleep plays a key role in the consolidation of memory. What has long been known for mammals, has only recently been discussed for non-mammalian species, including invertebrates like fruit flies and honeybees. One of the most prominent model organisms in memory research, the mollusk *Aplysia californica*, has recently gained attention by sleep research. With its relatively simple neuronal network comprising only ca. 20,000 neurons, the sea slug *Aplysia* embodies an ideal model for studies on the cellular and electrophysiological mechanisms underlying behavioural phenomena.

As in many other species, sleep was found essential for memory consolidation of operant conditioning in *Aplysia californica*. In the present work, the effect of sleep deprivation on consolidation of classical conditioning was explored.

A defensive reflex of *Aplysia*, the siphon withdrawal reflex, was classically conditioned. By pairing a trigger of the reflex with an electric shock, the reflexive withdrawal duration after training is prolonged. The study comprised two groups: the snails of one group were sleep deprived for 6 hours during the night after classical conditioning. The other group was allowed to sleep ad libitum and siphon withdrawal durations in both groups were assessed after 24 hours. This test was followed by an extinction training and both groups were retested 48 hours after conditioning training.

In the 24h-test, both groups showed prolonged withdrawal durations, so the performed training had successfully induced memory formation. Interestingly, unlike in former publications, the extinction training could not reverse the learned enhancement of the siphon withdrawal reflex. To the contrary, the learned change of behaviour was very robust and persisted for at least 3 days. As conditioning of *Aplysia*'s siphon withdrawal reflex always involves sensitization, supposedly the sensitization component was predominant here. This certainly very simple form of memory, which was induced by the applied training, did not profit from sleep during the consolidation phase, as the siphon withdrawal durations in both groups were not significantly different 24 hours after training. This is in line with findings

in other invertebrates and the hypothesis, that sleep is only beneficial for types of learning, which are more complex than classical conditioning.

*Aplysia californica* seems to be a very promising model organism to study the effect of sleep on learning. It is possible to electrophysiologically record from in vivo ganglia of the sea slug and to extract single cells for gene expression analysis. To further understand cellular processes during sleep, it might be interesting to compare alterations during sleep following classical and operant conditioning. In the former case, such processes are probably restricted to local potentiation of the monosynaptic connection, while the latter potentially involves replay or redistribution. *Aplysia* provides ideal conditions to correlate behavioural and molecular changes through sleep and might thus help to decipher the evolutionary conserved processes in memory formation during sleep.

# 5. Zusammenfassung

Schlaf spielt in der Gedächtniskonsolidierung eine entscheidende Rolle. Dies ist bei Säugetieren schon lange bekannt, wird jedoch erst neuerdings auch für Nicht-Säugetiere diskutiert, zum Beispiel bei Invertebraten wie Fruchtfliegen oder Honigbienen. Einer der bekanntesten Modellorganismen der Gedächtnisforschung, die Meeresschnecke *Aplysia californica*, findet zunehmend auch Beachtung in der Schlafforschung. Mit ihrem relativ einfachen neuronalen Netzwerk, das nur ca. 20.000 Neurone umfasst, verkörpert die Meeresschnecke *Aplysia* ein ideales Modell zur Erforschung von zellulären und elektrophysiologischen Mechanismen.

Wie in vielen anderen Spezies, wurde Schlaf bei *Aplysia californica* als essenziell für die Gedächtniskonsolidierung von operanter Konditionierung identifiziert. In der vorliegenden Arbeit wurde der Effekt von Schlafdeprivation auf die Konsolidierung klassischer Konditionierung untersucht.

Ein Abwehrreflex von *Aplysia*, der Siphonrückzugreflex, wurde klassisch konditioniert. Durch die zeitliche Koppelung von einem reflexauslösenden Reiz mit einem elektrischen Schock, ist die reflexartige Rückzugsdauer nach dem Training verlängert. Die Studie umfasste zwei Gruppen: die Tiere einer Gruppe wurden in der Nacht nach der klassischen Konditionierung für 6 Stunden am Schlaf gehindert, der anderen Gruppe wurde Schlaf nach Belieben gewährt. Die Siphonrückzugsdauer in beiden Gruppen wurden nach 24 Stunden erhoben. Auf diesen Test folgte ein Extinktionstraining und beide Gruppen wurden 48 Stunden nach dem Konditionierungstraining erneut getestet.

Im 24h-Test zeigten beide Gruppen verlängerte Siphonrückzugszeiten, das durchgeführte Training hatte also erfolgreich einen Lernprozess induziert. Interessanterweise konnte das Extinktionstraining, im Gegensatz zu früheren Publikationen, die gelernte Verstärkung des Siphonrückzugreflexes nicht rückgängig machen. Im Gegenteil, die gelernte Verhaltensänderung war sehr robust und hielt mindestens 3 Tage an. Da das Konditionieren des Siphonrückzugreflex von *Aplysia* immer eine Sensitivierung beinhaltet, dominierte hier vermutlich die Komponente der Sensitivierung. Die sicherlich sehr

einfache Gedächtnisform, die durch das angewendete Training hervorgerufen wurde, profitierte nicht von Schlaf während der Konsolidierungsphase, da sich die Siphonrückzugszeiten 24 Stunden nach dem Training in den beiden Gruppen nicht signifikant unterschieden. Dies ist in Einklang mit Erkenntnissen zu Schlafeffekten in anderen Invertebraten und der Hypothese, dass Schlaf nur Lernformen begünstigt, die komplexer als klassische Konditionierung sind.

Um den Effekt von Schlaf auf Lernen zu untersuchen, scheint *Aplyisa californica* ein vielversprechender Modellorganismus. Es ist möglich von in vivo Ganglien der Meeresschnecke elektrophysiologisch abzuleiten und einzelne Zellen für Genexpressionsanalysen zu extrahieren. Um zelluläre Vorgänge während des Schlafes besser zu verstehen, wäre es interessant, Veränderungen während dem Schlaf nach klassischer und operanter Konditionierung zu vergleichen. Im ersten Fall sind solche Prozesse vermutlich auf lokale Potenzierung der monosynaptischen Verbindung beschränkt ist, während im letzten möglicherweise Wiederholung oder Umverteilung eine Rolle spielen. *Aplysia* verfügt über ideale Eigenschaften, um Veränderungen durch Schlaf im Verhalten und auf molekularer Ebene zu korrelieren und könnte so helfen, die evolutionär erhaltenen Prozesse der Gedächtnisbildung während des Schlafes zu entschlüsseln.

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# 7. Eigenanteilserklärung

Die Arbeit wurde am Institut für medizinische Psychologie und Verhaltensneurobiologie unter Betreuung von Herrn Prof. Jan Born durchgeführt.

Die Konzeption der Studie erfolgte in Zusammenarbeit mit Herrn Albrecht Vorster, PhD Student.

Sämtliche Versuche wurden nach Einarbeitung durch Albrecht Vorster von mir eigenständig durchgeführt. Die Auswertung der Protokolle und des Videomaterials wurde von mir ausgeführt. Zur Verifikation wurden die Reaktionszeiten der einzelnen Tiere von Frau Verena Koppe blind erneut analysiert.

Die statistische Auswertung erfolgte nach Beratung durch das Institut für Biometrie und Anleitung durch Albrecht Vorster durch mich.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet habe. Sämtliche Grafiken und Bilder wurden von mir erstellt bzw. fotografiert.

Tübingen, den 04.09.2019

Kathrin Thiede

# 8. Veröffentlichungen

Inhalte dieser Dissertation wurden anteilig bereits im Rahmen folgender Publikation veröffentlicht:

Thiede, K. I., J. Born & A. P. A. Vorster (2021) Sleep and conditioning of the siphon withdrawal reflex in Aplysia. *The Journal of experimental biology*.

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