In search of the sixth sense- behavioural and structural studies of hygrosensation

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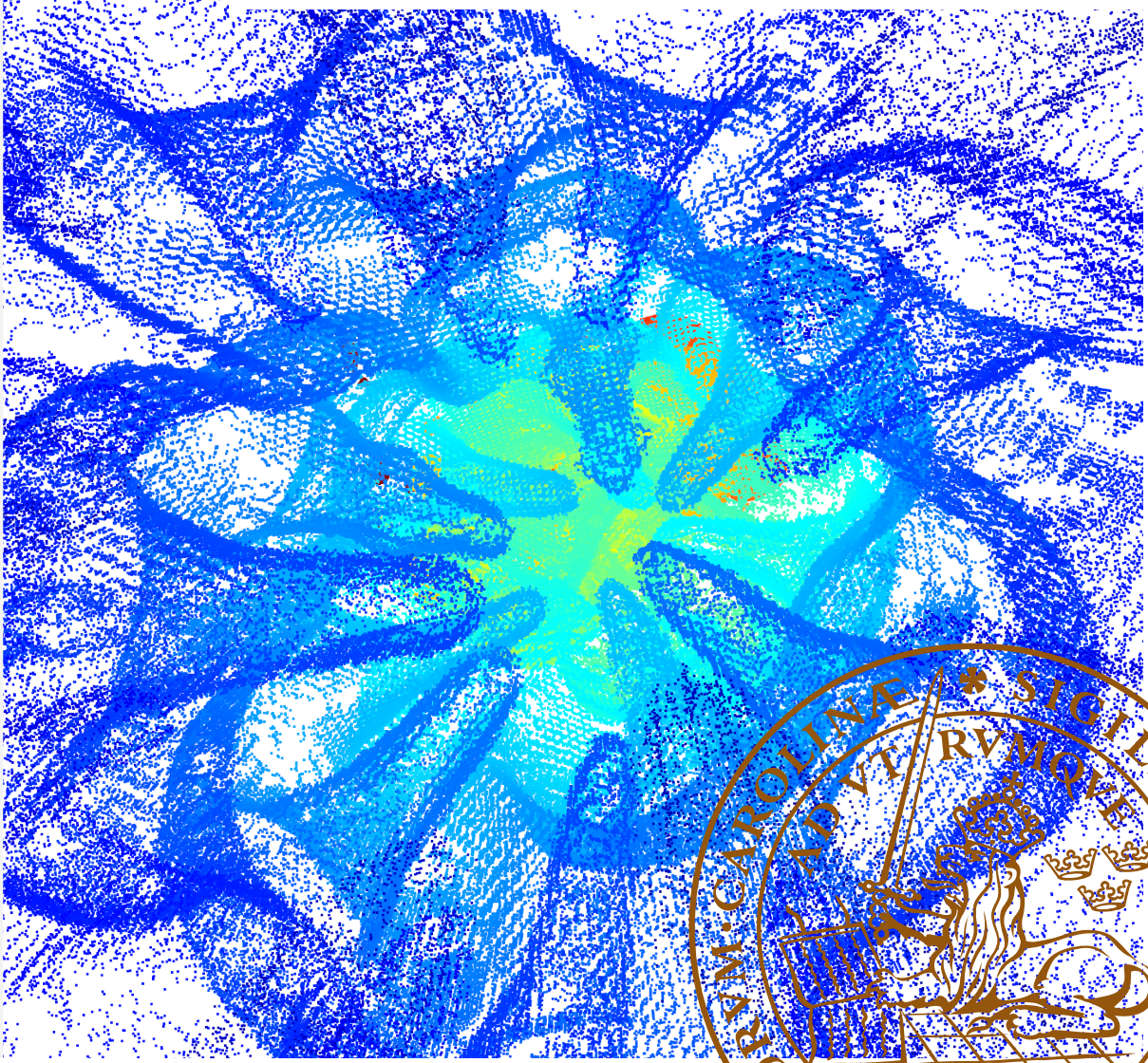

In search of the sixth sense- behavioural and structural studies of hygrosensation

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Ganesh Giri

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 14th of February at 08.00 in BMC I 1345, Department of Experimental Medical Science, Sölvegatan 19, Lund

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Abstract: Humidity is a critical environmental factor that significantly influences the behaviour of terrestrial organisms including humans. Despite its ecological importance, the field of hygrosensation remains less explored compared to sensory systems such as vision, olfaction, and touch. This thesis investigates the sensory phenomenon of hygrosensation in the fruit fly *Drosophila melanogaster*, focusing on how insects detect and respond to changes in environmental humidity. By integrating behavioural experiments, gene identification studies, and structural analyses, this work presents a comprehensive understanding of this essential sensory system. In the first study, a novel dynamic humidity arena was developed to examine humidity-guided behaviour in fruit flies. This arena provides real-time control over relative humidity (RH) with high precision, allowing for the observation of nuanced behavioural responses. Desiccated and starved flies exhibited a strong preference for 65–70% RH, while sated flies and humidity impaired mutants (Ir93a) showed no such preference. These findings highlight the influence of hydration status and the integrity of the hygrosensory receptor neurons on RH preference and establish the dynamic humidity arena as a versatile tool for behavioural studies in insects. The second study employed a comparative transcriptomic analysis of hygrosensory receptor neurons (HRNs) in *D. melanogaster* and *Aedes aegypti* to identify conserved genes that played a key role in hygrosensation. Behavioural studies using the dynamic humidity arena revealed the essential roles of the serotonin receptor *5-HT7*, transcription factor *nubbin*, and kinesin motor protein *Kif19A* in humidity-guided behaviour. The third study focused on the structural basis of humidity sensing, using serial block-face scanning electron microscopy (SBF-SEM) and a deep learning segmentation pipeline to analyse hygrosensilla ultrastructure under varying humidity conditions. Distinct chamber-specific differences in sensilla width and tapering patterns were observed between high and low RH conditions, suggesting potential structural adaptations. While these results support a mechanical transduction model of hygrosensation, further investigation with additional samples is needed to validate these findings.By investigating the behavioural, genetic and structural aspects of humidity sensation, this thesis lays a foundation for further exploration of the ecological and evolutionary significance of this critical sensory modality.

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Coverphoto by Ganesh Giri
Looking inside the sacculus, a 3D representation of the sacculus, created by using point clouds extracted from the contours of segmented EM images of a *Drosophila melanogaster* antenna. The conical projections are the sensilla located in the third segment of the sacculus

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Abstract

Humidity is a critical environmental factor that significantly influences the behaviour of terrestrial organisms including humans. Despite its ecological importance, the field of hygrosensation remains less explored compared to sensory systems such as vision, olfaction, and touch. This thesis investigates the sensory phenomenon of hygrosensation in the fruit fly *Drosophila melanogaster*, focusing on how insects detect and respond to changes in environmental humidity. By integrating behavioural experiments, gene identification studies, and structural analyses, this work presents a comprehensive understanding of this essential sensory system.

In the first study, a novel dynamic humidity arena was developed to examine humidity-guided behaviour in fruit flies. This arena provides real-time control over relative humidity (RH) with high precision, allowing for the observation of nuanced behavioural responses. Desiccated and starved flies exhibited a strong preference for 65–70% RH, while sated flies and humidity impaired mutants (Ir93a) showed no such preference. These findings highlight the influence of hydration status and the integrity of the hygrosensory receptor neurons on RH preference and establish the dynamic humidity arena as a versatile tool for behavioural studies in insects.

The second study employed a comparative transcriptomic analysis of hygrosensory receptor neurons (HRNs) in *D. melanogaster* and *Aedes aegypti* to identify conserved genes that played a key role in hygrosensation. Behavioural studies using the dynamic humidity arena revealed the essential roles of the serotonin receptor *5-HT7*, transcription factor *nubbin*, and kinesin motor protein *Kif19A* in humidity-guided behaviour.

The third study focused on the structural basis of humidity sensing, using serial block-face scanning electron microscopy (SBF-SEM) and a deep learning segmentation pipeline to analyse hygrosensilla ultrastructure under varying humidity conditions. Distinct chamber-specific differences in sensilla width and tapering patterns were observed between high and low RH conditions, suggesting potential structural adaptations. While these results support a mechanical transduction model of hygrosensation, further investigation with additional samples is needed to validate these findings.

By investigating the behavioural, genetic and structural aspects of humidity sensation, this thesis lays a foundation for further exploration of the ecological and evolutionary significance of this critical sensory modality.

Popular science summary

Have you ever noticed how humid weather can feel sticky and uncomfortable? While humans cannot directly sense humidity, we feel its effects through changes in the rate of sweat evaporation, which helps cool our bodies. To improve our comfort, we can use dehumidifiers, air conditioners, or better ventilation. For insects, however, humidity is far more critical—it is a matter of survival. Because insects are small and have a high surface area relative to their volume, they lose water quickly in dry environments, putting them at constant risk of dehydration. To counter this, they must monitor humidity in their surroundings and find conditions that match their physiological needs.

Insects rely on specialized structures called hygrosensilla to detect humidity. These tiny, hair-like structures, located on their antennae are well protected from external environment likely to protect them from physical damage. It is hypothesized that these hygrosensilla absorb or release moisture depending on the external humidity, which causes them to expand or contract, changing the pressure on sensory cells housed within. These cells relay signals to the insect's brain, which prompts it to react depending on its physiological needs. Using high-resolution electron microscopy, we visualized these remarkable structures and found that they expand significantly in humid conditions. This suggests that their size changes with environmental moisture, supporting the idea that they function like natural hygrometers. But it needs further validation to say for a fact that this indeed is the mechanism involved in humidity sensation.

To understand how insects behave in response to humidity, we developed a specialized experimental setup called the Dynamic Humidity Arena, where tethered fruit flies could explore different humidity zones depending on its trajectory. Experiments in this arena revealed that dehydrated or starving flies showed a clear preference for conditions with 65–70% relative humidity, likely as a survival strategy to replenish water. On the other hand, flies that were well-fed or had impaired humidity-sensing systems exhibited no specific preference, highlighting the role of physiological state and sensory capacity in driving humidity dependent behaviour.

We used single cell transcriptomics to investigate the genetic basis of humidity sensing. Scientists identified specific genes expressed in the cells responsible for sensing humidity in fruit flies. Flies with mutations in these genes displayed altered responses to sudden shifts in humidity when tested in the Dynamic Humidity Arena, confirming the importance of these genes in the sensory process. Interestingly, some of these genes are also present in mosquitoes, indicating that humidity sensing may have a shared evolutionary mechanism among insects.

This research is significant because it addresses a sensory system that remains relatively understudied compared to other senses like vision or smell. Understanding how insects sense and respond to humidity has the potential to solve real-world problems. For instance, by targeting the humidity-sensing mechanisms in mosquitoes, we could develop more effective repellents to curb the spread of diseases like malaria. Similarly, this knowledge could be leveraged to control pest insect populations, particularly those that depend on specific humidity and proximity to water for laying eggs.

Although much progress has been made, humidity sensing is a complex process that involves the interplay of molecular, structural, and behavioural factors. Future research will deepen our understanding of this fascinating sensory system and pave the way for innovative solutions to challenges in agriculture, pest control, and public health. In the tiny world of insects, where survival depends on sensing the unseen, humidity is not just a background factor—it is a lifeline.

List of Papers

Paper I

A dynamic humidity arena to explore humidity-related behaviours in insects

Ganesh Giri, Nicolas Nagloo and Anders Enjin

Journal of Experimental Biology, Volume 227, Issue 21, November 2024

Paper II

Conserved molecular signatures of hygrosensory neurons in two dipteran species

Kristina Corthals, **Ganesh Giri**, Johan Reimegård, Allison Churcher, Anders Enjin

Under review in Scientific Reports

Paper III

Humidity-dependent structural adaptations of *Drosophila melanogaster* hygrosensilla

Ganesh Giri, Anders Enjin

Under review in PLOS ONE

Abbreviations

RH	Relative humidity
HRN	Hygrosensory receptor neuron
np-sb	no-pore sensilla basiconica
np-sc	no-pore sensilla coeloconica
IR	Ionotropic receptor
PID controller	Proportional integral derivative controller
GUI	Graphical user interface
SBF-SEM	Serial block-face Scanning electron microscopy
IoU	Intersection over union
PCA	Principal component analysis
WMA	weighted moving average
FWHM	Full width half maximum
BDSC	Bloomington Drosophila Stock Center
MED	Multi Energy Deconvolution
RANSAC	Random Sample Consensus
p	Vapour pressure
H	Absolute humidity
TRPM8	Transient receptor potential melastatin-8
AMI	Acute myocardial infarction
COPD	Chronic obstructive pulmonary disease
THI	Temperature humidity index
RNAi	Ribonucleic acid interference
DH31	Diuretic hormone 31
AstC	Allatostatin C
sNPF	Short neuropeptide F
ORN	Olfactory receptor neurons
LFP	Local field potential

Introduction

Humidity in human health

Humidity plays a significant role in human health, influencing skin conditions, respiratory well-being, and the transmission of infectious diseases. Both low and high humidity levels have detrimental effects on health. Low humidity can lead to dry, rough, and itchy skin by increasing transepidermal water loss, while also promoting the formation of fine wrinkles due to reduced moisture and elasticity. Conversely, high humidity, although it may improve skin hydration, can impair the natural shedding of dead skin cells, contributing to skin roughness and potentially exacerbating conditions like eczema [1].

Respiratory health is also strongly influenced by humidity. When relative humidity (RH) falls below 40%, it promotes the survival and transmission of respiratory viruses by facilitating the formation of smaller, more stable droplet nuclei that remain airborne longer. Additionally, the drying effect on the respiratory tract weakens its defences, increasing susceptibility to infections. High humidity levels, on the other hand, above 80%, foster the growth of bacteria and fungi, which can increase the risk of respiratory infections and exacerbate conditions like asthma [2,3]. Maintaining an indoor RH range of 40–60% is generally recommended to support optimal health, as it minimizes pathogen survival, prevents excessive bacterial growth, and supports both skin and respiratory barrier functions [2,4].

The relationship between humidity and the transmission of respiratory viruses is elaborate and varies by virus type. For enveloped viruses like influenza, measles, and SARS-CoV-2, higher humidity levels above 50% generally reduce infection risk by destabilizing these viruses. Non-enveloped viruses, such as rhinovirus and adenovirus, tend to survive longer at higher humidity levels (70–90%), but the infection risk increase from humidifying air to around 50% RH is minimal for these viruses. This variability highlights that there is no single RH level that minimizes transmission risk for all viruses. Instead, increasing ventilation rates emerges as a consistently effective strategy for reducing infection risk across most virus types [5].

Given its impact on health, humans have developed various strategies to adapt to and regulate humidity levels in indoor environments. In dry conditions, humidifiers are often used to add moisture to the air, alleviating skin dryness and respiratory irritation. Conversely, in regions with high humidity, dehumidifiers and air conditioning systems are employed to reduce excess moisture, preventing mold growth and bacterial proliferation. Architectural designs, incorporating ventilation

systems and insulation materials, also help regulate indoor humidity, providing a balance that enhances comfort and reduces health risks associated with extreme humidity conditions. Therefore, understanding more about humidity and its effects is crucial for developing targeted solutions that not only improve human comfort but also safeguard against health risks, including the spread of infectious diseases. Despite the importance of humidity in managing human health, we lack dedicated sensory cells that can directly detect changes in humidity [6]. Instead, we rely on other indirect methods to monitor and adapt to these fluctuations.

Wetness sensation in humans

The perception of wetness in humans is another essential response to humidity. Wetness perception integrates both thermal and mechanical cues [7,8] and plays a vital role in tactile dexterity, particularly in precision tasks like holding a pen or lifting objects. Moisture at the skin-object interface influences friction, requiring fine-tuned grip force adjustments to prevent slipping. Mechanoreceptors in the skin detect subtle changes, such as microslips, enabling rapid, unconscious corrections to maintain a secure hold. It has traditionally been thought that humans lack dedicated hygroreceptors in the skin, with wetness perception relying instead on the brain's ability to integrate signals from touch and temperature changes caused by contact with moisture. However, emerging research points to a more complex mechanism involving the transient receptor potential melastatin-8 (TRPM8) channel, a cold-sensitive ion channel in the skin, as playing a critical role in this process [8].

Thermal cues, particularly cooling sensations, are crucial for wetness detection. As moisture evaporates from the skin, the associated drop in temperature signals wetness. This process is so sensitive that people may perceive wetness even in dry, cold conditions that simulate the cooling effect of moisture. interestingly, even in the absence of moisture, cold-dry stimuli that fall within the activation range of TRPM8 (28–15°C) can mimic the cooling associated with evaporation, leading to a strong sensation of wetness. This response varies across different parts of the body, corresponding to regional variations in sensitivity to cold [8]. These findings suggest that TRPM8 acts as a peripheral sensor that contributes significantly to the perception of wetness. TRPM8 can be activated chemically, independent of temperature changes. Menthol, a compound known to stimulate TRPM8, can produce a distinct sensation of wetness without lowering skin temperature. Remarkably, the intensity of wetness perceived from menthol is greater than that caused by skin cooling or direct contact with moisture-containing substances [8]. This highlights TRPM8's dual role in detecting both cold and wet stimuli, a function that mirrors similar mechanisms found in other insects, where sensors for cold and humidity are often coexpressed [9].

Mechanical cues are particularly important when cooling sensations are absent, as in the case of warm wetness, where the stickiness and texture of fluids interacting with the skin provide essential information. Mechanical interactions such as light touch, friction, and pressure further enhance wetness perception. For example, increased stickiness or light pressure improves the ability to detect wetness, while reduced contact, such as during sweating, can diminish this sensation [6].

At the neurophysiological level, wetness perception results from the integration of signals from two types of sensory fibers: Aδ thermoreceptive fibers, which detect cold, and Aβ mechanoreceptive fibers, which sense touch and pressure. These sensory signals are transmitted through the peripheral nervous system, reaching the thalamus for preliminary processing, before being relayed to the somatosensory cortex and the insular cortex, where the brain integrates these inputs to form a coherent perception of wetness [6]. Additionally, prior experiences play a critical role in shaping wetness perception. The brain constructs a neural template based on past exposure to wet stimuli, allowing it to filter and interpret new sensory information. This multisensory integration highlights the close relationship between wetness and humidity perception. The sensitivity of humans to wetness is significant, as even a small drop of water (as little as 20 μl) can be detected. This heightened sensitivity is essential for maintaining thermoregulation, water balance, and object manipulation. Deficits in wetness perception, such as those caused by nerve damage or conditions like multiple sclerosis, emphasize the importance of this sensory function in everyday life [6].

Impact of Humidity on Immune Health

Humidity plays a critical role in maintaining immune health by influencing respiratory defence mechanisms, epithelial integrity, and the prevalence of environmental allergens and pathogens. Deviations from the optimal relative humidity (RH) range of 40–60% can significantly compromise the immune system, increasing vulnerability to infections and allergies [10].

One primary way RH affects immunity is by altering mucociliary clearance, a vital defence mechanism of the respiratory system [11–13]. This process relies on mucus to trap foreign particles and pathogens, which are then cleared by the coordinated movement of cilia. When RH is too low, the mucus becomes thick and viscous, impairing the cilia's ability to effectively remove it. This results in mucus build-up, creating an environment where infections are more likely to occur. On the other hand, excessively high RH fosters damp conditions that encourage the proliferation of harmful microorganisms, such as mold, bacteria, and viruses, further increasing health risks.

The integrity of the epithelial barrier, a critical line of defence in the respiratory tract, is also impacted by RH levels [14]. Low RH can cause dryness and irritation, weakening the epithelial layer and increasing its permeability. This disruption

allows allergens, pollutants, and pathogens to penetrate deeper into the body, potentially triggering inflammation. Damaged epithelial cells may release mediators like alarmins, which exacerbate immune responses and contribute to respiratory issues [15].

Humidity also influences allergic sensitization by altering the levels of allergens in the environment [15]. High RH provides favourable conditions for the growth of dust mites and mold, common allergens that can trigger immune responses in sensitive individuals. Meanwhile, dry air can increase airborne pollen concentrations, leading to symptoms such as sneezing, nasal congestion, and wheezing. Both low and high RH levels affect the survival and transmission of pathogens. For instance, low RH enhances the stability and aerosol transmission of viruses like influenza, enabling them to remain viable for longer periods in the air. In contrast, high RH promotes the growth of bacteria such as *Legionella*, which thrive in moist environments [15]. These dynamics demonstrate how humidity can influence not only individual susceptibility but also broader patterns of disease transmission and the importance of balanced humidity for managing allergy-related health issues.

The role of humidity in health and disease transmission

Humidity plays a multifaceted role in influencing human health and disease transmission, affecting physiological processes, disease susceptibility, and the spread of infectious agents. Several theories have been proposed to explain the ways in which humidity impacts health, with its effects spanning cardiovascular, pulmonary, and infectious diseases, as well as the dynamics of disease transmission.

Humidity is a key determinant in heat stress, particularly in hot climates, where high levels impair the body's ability to cool itself by reducing the efficiency of sweat evaporation. This can lead to elevated core body temperature and increase the risk of heat-related illnesses such as heat stroke. Conversely, low humidity, typical of colder months, can exacerbate health risks by accelerating heat loss, causing respiratory strain, and impairing temperature regulation.

In terms of cardiovascular health, high humidity can burden the system by hindering perspiration and temperature homeostasis, potentially resulting in increased heart rate and respiratory fatigue. Such conditions may exacerbate ailments like angina. Conversely, lower humidity, often accompanied by cold temperatures, has been associated with a higher incidence of acute myocardial infarction (AMI). This link is partly attributed to the interplay between respiratory infections and influenza, which are more prevalent in low-humidity conditions and are known triggers for cardiovascular events.

Pulmonary health also exhibits sensitivity to humidity levels. Low humidity promotes the survival and transmission of respiratory viruses such as influenza,

which thrive in dry air and can spread more readily during colder months. Additionally, dry conditions can compromise the nasal mucosa, weakening its barrier function and facilitating the entry of infectious agents. High humidity, however, fosters bacterial and fungal growth, which can aggravate asthma symptoms and increase the prevalence of respiratory infections due to indoor crowding, mold, and allergens common in warm, moist environments.

The role of humidity extends beyond human health to its impact on disease transmission, particularly in respiratory and insect-borne diseases. Relative humidity (RH) plays a key role in the transmission dynamics of infectious diseases, especially those spread through respiratory droplets. These droplets, expelled during coughing, sneezing, or speaking, can carry pathogens, and RH influences how long these droplets stay suspended in the air and how long the pathogens remain viable. At low RH levels, common in indoor environments during winter or in controlled spaces like airplanes, droplets evaporate quickly, becoming smaller and lighter [3]. This process prolongs the airborne duration of pathogens, increasing the likelihood of disease transmission. For example, respiratory viruses such as influenza are more stable in environments with low RH (20–50%), while some pathogens thrive at higher RH levels (80%).

Low RH not only increases the airborne transmission of pathogens but also compromises human defences by drying out the nasal mucous membrane, impairing its barrier function and facilitating the entry of infectious agents. On the other hand, high humidity fosters bacterial and fungal growth, exacerbating conditions like asthma and increasing the risk of respiratory infections [16]. Small aerosol particles, typically 1–10 μm in size, are less affected by RH compared to larger droplets, as they are primarily influenced by airflow patterns and Brownian motion.

These theories and observations emphasize the dual nature of humidity's impact on health, where both high and low levels pose distinct risks depending on the specific context. High humidity can strain cardiovascular and respiratory systems, promote microbial growth, and support insect-borne disease transmission, while low humidity facilitates viral spread, impairs the body’s defences, and increases susceptibility to respiratory illnesses. Maintaining an optimal RH range, around 50%, is critical for minimizing health risks and curbing the transmission of infectious diseases. However, RH still plays a critical role in droplet behaviour and the persistence of infectious particles in the air. Maintaining an optimal RH range of around 50% is crucial for minimizing the survival and spread of infectious droplets, thus helping to reduce the risk of respiratory diseases [17].

Effect of Humidity on People with Pre-Existing Conditions and Vulnerable Groups

Humidity has a complex and variable impact on individuals with pre-existing conditions, such as cardiovascular and pulmonary diseases, as well as on vulnerable groups like children and the elderly. These effects are influenced by factors such as humidity level, temperature, geographic location, and individual acclimatization, highlighting the need for tailored approaches to managing humidity exposure.

For individuals with cardiovascular disease, the relationship with humidity remains inconclusive. A study conducted in twelve US cities found no link between humidity and hospital admissions for heart disease or myocardial infarction [18]. However, research on acute myocardial infarction (AMI) presents mixed results. In western Sicily, the relationship between relative humidity and angina showed mixed results, with lower humidity levels being negatively associated with angina hospital admissions, while an increase in minimal humidity was linked to higher admissions in males and the overall population [19]. On the other hand, a Japanese study of over 87,000 AMI patients associated lower humidity with increased hospitalizations [20]. The findings suggest that low humidity might exacerbate cardiovascular risks, especially for elderly populations in regions like Japan, where protective measures against dry air are recommended.

Pulmonary diseases, including asthma and chronic obstructive pulmonary disease (COPD), also show variable responses to humidity [21]. Studies on COPD highlight the complexity of these interactions: in Taiwan, lower humidity during winter was linked to more exacerbations [22], whereas a study in Istanbul found no relationship between humidity and COPD outcomes [23]. In Shanghai, high humidity was shown to amplify the adverse effects of low temperatures on COPD patients, with risks significantly increasing when relative humidity exceeded 70% [24]. Similarly, asthma research reflects regional differences. For instance, asthma incidence in Trinidad increased with higher humidity during the wet season, while in Japan, low humidity was linked to increased paediatric asthma admissions [21]. These findings underline the interplay between humidity, temperature, and other environmental factors in respiratory health and points out that we still do not completely understand the ways in which humidity might affect our health.

Children, as a particularly vulnerable group, are significantly affected by humidity due to its role in climate-sensitive conditions such as respiratory illnesses, diarrheal diseases, and allergic reactions. For example, low humidity has been linked to higher cases of rotavirus diarrhoea [25], while high humidity correlates with increased hand, foot, and mouth disease infections [26]. Humidity also exhibits both positive and negative effects on childhood asthma and allergic diseases, with regional and seasonal differences playing a major role [27]. The expected increase in humidity levels due to climate change poses an additional risk, potentially exacerbating these conditions in children.

The elderly, like children, are sensitive to changes in humidity, though the impact varies based on acclimatization and geography. In Sweden and colder regions of the U.S., higher humidity has been associated with increased mortality [21,28], whereas in Brisbane, Australia, humidity had little effect, likely due to the elderly population's adaptation to warm, humid conditions [21]. This suggests that the elderly in warmer climates may develop a tolerance to high humidity, mitigating its adverse effects.

Overall, the relationship between humidity and health outcomes for individuals with pre-existing conditions and vulnerable groups is multifaceted and regionally specific. Factors such as geography, local climate, air quality, and socioeconomic conditions further complicate this relationship. These nuances underscore the importance of further research to develop evidence-based recommendations for managing humidity exposure, ensuring the health and well-being of these at-risk populations.

The Overlooked Impact of Humidity on Insect-Borne Diseases

Humidity also plays an often-overlooked role in the ecology of insect-borne diseases. Vectors like mosquitoes, ticks, and flies transmit a range of infectious diseases, such as malaria, dengue fever, Lyme disease, and leishmaniasis. While temperature is often studied as a major factor influencing the spread of these diseases, humidity is just as critical, affecting both the survival and behaviour of disease vectors. For mosquitoes, environmental humidity influences every stage of their lifecycle and their ability to transmit pathogens. Anopheles mosquitoes, responsible for malaria, and Aedes mosquitoes, which transmit diseases like dengue and Zika, are highly sensitive to humidity. High humidity extends their lifespan and increases the likelihood of pathogen transmission, while low humidity can stress mosquitoes, reducing their fitness and altering their behaviour. The combined effects of humidity and temperature can either exacerbate or mitigate these stresses [16].

Beyond its impact on vector biology, humidity also influences pathogen survival in insect vectors. For example, humidity plays a critical role in the evaporation and sedimentation of respiratory droplets, as discussed earlier. In insects, low humidity can severely limit their ability to survive and reproduce, reducing the spread of diseases like Lyme disease, malaria, and dengue. Some pathogens, such as those causing malaria, show a strong correlation between RH and disease outbreaks. For example, in India, malaria epidemics have been found to correlate more strongly with RH than with temperature or rainfall [29]. This highlights the significant role humidity plays not only in vector biology but also in pathogen survival and transmission dynamics.

Relevance of studying the effects of humidity

Understanding the effects of humidity on various biological systems, particularly in relation to vector biology and disease transmission, is of paramount importance for public health. Humidity plays a significant role in shaping the behaviour and survival of disease vectors, such as mosquitoes, ticks, and flies, which are responsible for transmitting a range of infectious diseases. By incorporating humidity data into predictive models, public health experts can more effectively forecast disease outbreaks and design targeted interventions. For example, vector control strategies could focus on manipulating local humidity levels to reduce the survival rates of these vectors, while public health measures like using dehumidifiers or improving ventilation in areas where vectors thrive can help mitigate the spread of insect-borne and airborne diseases. Therefore, in addition to temperature, humidity should be considered a critical factor in managing the spread of infectious diseases globally.

In this context, studying the effects of humidity on animals, especially insects, is key to advancing our understanding of its broader biological impact. Insects possess an extraordinary ability to detect and respond to changes in humidity, which serves as an essential part of their behaviour and survival. Unlike humans, who rely on complex multisensory integration to detect humidity changes, many insects have specialized sensory structures that allow them to sense even minute shifts in moisture levels. This "sixth sense" enables insects to find optimal humidity conditions and adjust their behaviour accordingly. By studying the physiological and neural mechanisms behind this remarkable ability, we can gain valuable insights into how animals, including humans, respond to environmental moisture changes. This knowledge can then be applied to improve human health, particularly in areas such as skin care, respiratory health, and the management of vector-borne diseases. Furthermore, by unravelling the intricacies of insect hygrosensation, we open the door to innovative strategies for controlling humidity-related health issues and improving disease prevention efforts. Ultimately, understanding the way insects perceive humidity can offer valuable lessons for managing the environmental factors that influence human well-being.

Humidity - an important abiotic factor for terrestrial life

Humidity is a vital abiotic factor that profoundly influences the survival, physiology, and distribution of terrestrial animals [30]. It plays a central role in maintaining water balance, regulating body temperature, and supporting essential life processes such as reproduction [31]. Most animals have a specific range of humidity that is ideal for their survival, comfort and align with their physiological needs. However, both extremes of humidity can be detrimental. Saturated air

promotes fungal and bacterial growth, negatively affecting the organism [32,33], while low humidity can lead to desiccation, especially in species lacking efficient water conservation mechanisms [34].

Humidity, in conjunction with temperature, plays a vital role in shaping the survival strategies of terrestrial animals, particularly insects. This interplay directly influences critical physiological processes, including body temperature regulation and cold resistance. Insects in dry, warm conditions utilize evaporative cooling to regulate their body temperatures, preventing overheating in high-temperature environments. For instance, honeybees employ evaporative cooling to maintain their colony's temperature, lowering it by 5–8°C below the ambient temperature [35]. Similarly, cold resistance in some insects improve under reduced water content, a crucial adaptation for surviving freezing conditions. The grasshopper *Chortophaga viridifasciata* exemplifies this strategy, gradually decreasing its water content to about 65% during the fall [35]. This reduction minimizes the amount of freezable water in its tissues, enhancing its ability to withstand winter temperatures.

While dry conditions enable evaporative cooling, reduced water content improves cold resistance, showcasing the diverse adaptations insects have evolved to thrive under varying environmental conditions. These adaptations ultimately shape species' ecological niches and determine their distribution across different environments.

Ecological importance of humidity

The Earth’s atmosphere is composed of a mixture of gases, primarily nitrogen and oxygen, with smaller amounts of other gases, such as carbon dioxide, argon, and water vapor. Although water vapor represents only a minor fraction of atmospheric gases by volume, it plays a disproportionately important role in both environmental and biological processes. Unlike other atmospheric gases, water vapor is distinct due to its variability and sensitivity to temperature [36]. As temperature rises, air holds more water vapor, which causes humidity levels to increase. This concentration of water vapor is influenced by factors such as temperature, altitude, and geographic location, resulting in considerable fluctuations in atmospheric humidity across regions and seasons.

These fluctuations in humidity have shaped unique ecological landscapes, creating specific environments where various species have adapted and thrived. In areas with consistently high humidity, such as tropical rainforests, organisms have evolved to withstand and even depend on moisture-rich conditions; plants and animals in these regions often have specialized adaptations for deterring pathogens, cooling, or heat dissipation. In contrast, arid or semi-arid environments characterized by low humidity have led to the evolution of drought-resistant flora and fauna that conserve water efficiently. Such diverse habitats highlight the influence of atmospheric

humidity on biodiversity, enabling species to evolve in ways that align with their environmental moisture conditions. Humidity, therefore, is not only a factor in weather patterns but also a crucial driver of ecological diversity and species specialization across the globe.

Geographically, relative humidity levels are higher in coastal areas due to the proximity to large water bodies, which continuously supply moisture to the air, creating humid environments that support a diverse range of organisms. In regions with dense vegetation, relative humidity levels are similarly elevated as plants release water vapor through transpiration—a process where water travels from roots through stems and evaporates from leaves [37]. This natural moisture cycle enhances local humidity, fostering habitats ideal for organisms adapted to humid conditions. By contrast, in drier, inland, or desert regions where RH levels are generally lower, less atmospheric moisture creates arid habitats that support specialized flora and fauna adapted to these challenging conditions.

Understanding Humidity: Definitions and Measurement Metrics

Given its profound influence on ecosystems, climate, and biological processes, a clear understanding of what humidity is and how it is measured becomes essential. Humidity, in a general sense, refers to the amount of water vapor present in the air, but this can be measured in several ways. One of the most straightforward ways to quantify humidity is by measuring absolute humidity (H), which defines the mass of water vapor (mH_2O) per unit volume of air (V_{net}) [38]

$$H = \frac{mH_2O}{V_{net}}$$

While absolute humidity provides a straightforward measure of the water vapor content in the air, it does not give the full picture of how humidity interacts with materials and environments. In many contexts, what matters most is the rate at which water evaporates, as this rate can influence the properties and stability of various materials, from soil and plants to fabrics and biological tissues. This evaporation rate is not solely dependent on the water vapor concentration but is also affected by factors like temperature and the saturation of the air.

To understand this dynamic, relative humidity (RH) is a critical metric. RH represents the percentage of water vapor in the air relative to the maximum amount the air can hold at a given temperature [38]. It is calculated as:

$$Relative\ Humidity\ (RH) = \left(\frac{Actual\ Vapour\ Pressure}{Saturation\ Vapour\ Pressure}\right) \times 100$$

Vapor pressure refers to the pressure exerted by water vapor in the air. The actual vapor pressure represents the partial pressure exerted by the existing amount of water vapor, while the saturation vapor pressure indicates the maximum pressure exerted by water vapor when the air is fully saturated at a given temperature. This concept is visualized in the vapour pressure vs temperature plot (Figure 1 A) calculated by using a simplified version of the Clausius-Clapeyron equation [39], where the saturation vapor pressure (p) increases exponentially with temperature (T in °C), showing how warmer air can hold more moisture.

$$p = 6.1094\ exp\left(\frac{17.625\ T}{243.04 + T}\right)$$

The plot also includes curves for actual vapor pressures at different RH levels, demonstrating how RH is a ratio of actual to saturation vapor pressure. When the actual vapor pressure equals the saturation vapor pressure (RH = 100%), the air is fully saturated, and condensation may occur.

This relationship has practical implications for evaporation. As shown in the evaporation rate vs RH curve (Figure 1B), evaporation rates decrease at high RH levels because the air is already saturated and cannot absorb much more moisture. Conversely, at low RH levels, the air has a greater capacity to absorb moisture, leading to faster evaporation. These dynamics are critical for understanding processes like drying, hydration, and condensation in both natural and controlled environments. For instance, the second plot clearly illustrates how evaporation slows dramatically as RH approaches 100%, which can influence the ability to dissipate heat by evaporative cooling in animals.

Evaporation rates are influenced by the combined effects of temperature and RH. High temperatures enhance evaporation by increasing the energy available for water molecules to transition into the vapor phase. Simultaneously, low RH levels create a greater capacity for the air to absorb water vapor, further accelerating the evaporation process. In contrast, low temperatures and high RH levels suppress evaporation due to reduced molecular energy and the limited ability of saturated air to hold additional moisture. To provide a visual representation of this interplay, the 3D plot (Figure 1 C) demonstrates how evaporation rates vary across a range of temperatures and RH levels. The surface plot clearly shows that evaporation rates peak under conditions of high temperature and low RH, while they diminish significantly at low temperatures and high RH.

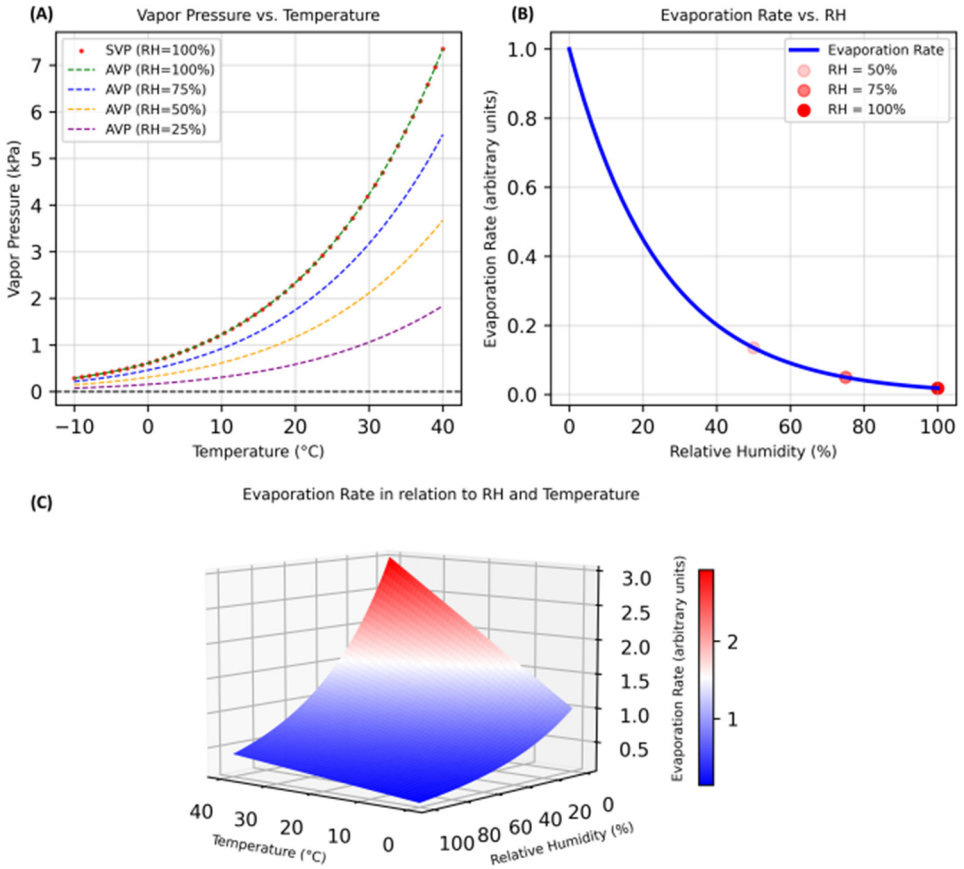

Figure 1: - Dynamics of relative humidity. (A) Vapor pressure vs. temperature curve illustrating the exponential increase in vapor pressure with temperature. Solid red dots represent saturation vapor pressure, while dashed lines indicate actual vapor pressure, calculated as the product of RH and saturation vapor pressure. (B) Relationship between evaporation rate and RH, modelled using an exponential decay function. The evaporation rate decreases significantly as RH increases. (C) A 3D plot depicting the interplay between temperature, RH, and evaporation rate. Evaporation rate peaks at high temperatures and low RH and diminishes under opposite conditions.

Impact of relative humidity on plants and animals

RH significantly influences the lives of plants, animals, and their surrounding ecosystems. In regions with low RH, dry conditions prevail, increasing the likelihood of wildfires and droughts, which can disrupt ecosystems and threaten biodiversity. Conversely, high RH contributes to the formation of clouds, fog, and precipitation, creating moisture-rich environments that support water-dependent organisms. Soil conditions are also closely tied to RH levels; higher RH helps soils retain moisture for extended periods, benefiting plant growth and providing

sustenance for soil-dwelling organisms. However, excessively high RH can result in waterlogging, negatively impacting soil health and plant stability, while low RH can hasten soil drying, making it harder for plants to meet their hydration needs [40,41].

Plant transpiration, crucial for water and nutrient regulation, is directly affected by RH [42]. When RH is low, the rate of transpiration increases as the water vapor concentration difference between the leaf and the surrounding air widens. This can result in significant water loss from plants, risking wilting if water uptake cannot keep pace. In contrast, high RH slows transpiration, conserving water within the plant but potentially reducing nutrient transport efficiency. Through these effects, RH directly shapes the physical environment, supporting diverse habitats that allow a variety of organisms to thrive in different ecosystems.

For animals, RH impacts behaviour and survival. For instance, geckos benefit from high RH as it enhances the adhesive properties of their setae, allowing them to maintain a secure grip on surfaces. This effect arises from the softening of β-keratin in humid conditions [43]. Furthermore, foraging behaviour in Yellow pine chipmunks is directly influenced by humidity. They experience improved olfactory detection of buried seeds in moist conditions due to increased volatilization of seed odors, enhancing their foraging success [44].

Behavioural Adaptation to humidity in animals

Animals display a wide range of behavioural adaptations to manage the challenges posed by humidity, often by selecting microhabitats or adjusting their body posture to regulate water loss and heat dissipation effectively. These strategies help minimize the energy costs of maintaining body temperature, especially in environments with fluctuating humidity levels

One common adaptation is posture adjustment to control the surface area of the body exposed to the environment [45]. By altering their surface-to-volume ratio, animals can modulate their heat dissipation. For example, titi monkeys adopt heat-dissipating postures as ambient temperatures rise above 27 °C and also respond to increased humidity at specific temperatures by changing their posture. However, when humidity levels exceed 80% at 24 °C, these monkeys reduce their use of such postures, likely balancing the need for cooling against the risk of excessive water loss [45].

Relative humidity significantly impacts evaporative cooling, influencing behaviours such as sun avoidance and habitat selection. For instance, baboons tend to avoid sunny areas in humid conditions to minimize water loss, while isopods in littoral zones, like *Ligia*, prefer highly humid environments, avoiding conditions that would increase evaporation. In dry environments, intertidal isopods like *Campecopea*

hirsuta curl their bodies to conserve moisture. Similarly, terrestrial isopods aggregate in sheltered environments using behaviours like thigmokinesis and thigmotaxis, reducing water loss and offering protection from predators [46].

Other species adapt their hydration behaviours in response to combined humidity and temperature stresses. For example, goats adjust their feeding and rumination times relative to the temperature-humidity index (THI), increasing water intake and reducing activity during high THI conditions [47]. Likewise, pigs increase respiratory frequency and urination on solid floors when exposed to high temperatures and humidity, possibly due to space constraints that prevent more effective cooling behaviours [48].

The intensity of an animal's response to humidity often correlates with the degree of water loss they experience. Isopods from mesic habitats show distinct humidity-driven behaviours, including a preference for light under dehydrated conditions, which may help them find moisture [46]. These adaptations showcase the intricate interplay between environmental humidity and behavioural responses, enabling animals to maintain hydration and thermoregulation in diverse habitats.

Importance of RH for insects

Humidity plays a vital role in ensuring the comfort and health of large animals, but for smaller animals such as insects, it is even more crucial due to their small size and large surface area to volume ratio, which makes them highly susceptible to desiccation [34,49]. This vulnerability arises from the increased potential for water loss through their cuticle, respiration, and excretion. Insects rely on ambient RH to minimize these losses and maintain their water balance. Without an optimal humidity environment, they face significant risks of dehydration and desiccation, which can be fatal. While insects have evolved various adaptations such as specialized cuticular lipids, spiracular control, and behavioural strategies to mitigate water loss, these mechanisms often depend on the presence of sufficient environmental humidity to be effective [50–52]. Consequently, ambient RH is not just a factor of comfort but a critical determinant of survival for insects.

Humidity significantly influences insect behaviour, particularly in foraging, oviposition, and other ecological interactions. Floral humidity, a phenomenon where the humidity in the headspace of a flower is elevated due to nectar evaporation and floral transpiration, plays a critical role as a foraging cue. Pollinators such as hawkmoths, bumblebees, and flies can detect and respond to floral humidity [53,54]. Insects often prefer flowers with higher floral humidity levels, even when nectar rewards are absent, indicating that floral humidity can signal the potential presence of nectar. Factors such as petal surface area, cuticle permeability, and nectar volume affect the reliability of floral humidity as a nectar

indicator, with xeric environments making this cue particularly vital due to water scarcity [53,54].

Humidity also guides oviposition behaviours in insects, such as mosquitoes, which use humidity cues to locate suitable water sources for laying eggs [55]. Beyond foraging and oviposition, insects demonstrate remarkable sensitivity to humidity for other purposes. Honeybees, for example, actively regulate humidity within their nests to create optimal conditions for brood development by engaging in behaviours like fanning to reduce humidity or collecting water and evaporating nectar to increase humidity [56]. *Triatoma infestans* utilize humidity cues along with other signals to locate hosts, while an insect's physiological state, such as water balance, can influence its attraction to humid environments or avoidance of dry conditions [57].

Internal state and its impact on humidity dependent behaviour

Internal state refers to the condition of an animal arising from its physiological processes, including developmental stage, nutritional or mating status, and sensory information processing. This state interacts with external environmental stimuli to produce context-dependent behaviours that align with the animal's biological goals [58]. In the context of humidity, internal state plays a pivotal role in shaping insect behaviour, influencing critical activities such as oviposition, foraging, and hydration maintenance.

For instance, in mosquitoes, internal physiological changes associated with blood meals and egg development significantly impact humidity-dependent behaviour. Blood-fed, gravid mosquitoes become less responsive to host-seeking cues and are instead strongly attracted to moisture cues, such as water vapor and visual indicators of standing water, to identify suitable oviposition sites [55,58–60]. At these sites, they further evaluate water salinity using tarsal sensory neurons to ensure optimal conditions for larval development. Similarly, low ambient humidity can drive an increase in blood-feeding frequency in *A. aegypti*, linking hydration status to feeding behaviour [61].

In pollinators, ambient humidity influences foraging decisions, reflecting the internal balance between energy and water needs. *Manduca sexta* moths exposed to low humidity (20%) consume more water but have shorter lifespans compared to those in more humid environments, demonstrating how osmotic stress impacts survival and behaviour [62]. These observations suggest that dry conditions may even prompt migrations to more humid habitats in search of better conditions.

By filtering and processing sensory information, internal state enables insects to adapt their behaviours to achieve essential goals such as reproduction, hydration,

and survival. Therefore, a dedicated set of organs and structures are required for sensing external humidity and integrating this information with the insect's internal state to drive humidity dependent behaviour ensuring the survival of the insect.

Hygrosensory structures in insects

Since humidity plays such a crucial role in the life of insects, it is natural that they have evolved organs and structures that are able to precisely detect RH changes in their environment. Insects possess sensory neurons specifically responsive to humidity changes, known as hygrosensory receptor neurons (HRNs) [63]. These neurons were first studied in the antennae of honey bees (*Apis mellifera*). HRNs are housed within hair-like structures called hygrosensilla (singular: sensillum), which are typically found on the antenna [63–65]. Within the hygrosensilla there are three distinct types of neurons working in unison, forming a hygrosensory triad. These neurons include one moist cell (responding to increases in humidity), one dry cell (responding to decreases in humidity) and one hygrocool cell (responding to cooling). The HRNs are exceptionally sensitive to changes in RH and in *A. mellifera* and *M. sexta* they are reported to respond to changes as low as 1% RH [66,67].

The hygrosensilla in various species exhibit diverse structural adaptations but can be broadly classified as a stubby and conical protuberances. In *A. mellifera*, hygrosensilla are located on the flagellum, the elongated, segmented part of the antenna beyond the scape and pedicel. Each sensillum is set within a shallow, circular depression in the cuticle that features a central opening. From this opening, a mushroom-shaped protrusion with an irregularly shaped head emerges, sitting just below the antennal surface [68]. In cockroaches *Periplaneta americana*, hygrosensilla, also termed as sensilla capitula, are located on their antennae, specifically on the distal part of alternating segments of the flagellum and on each segment of some distal meristal segments. These sensilla are typically surrounded by trichoid and basiconic sensilla, along with long bristles, which likely provide protection from environmental factors [69]. In the stick insect *Carausius morosus*, the hygrosensillum is a small, smooth cuticular peg positioned at the base of a pit. It does not extend beyond the enclosing walls, which form a dome-like structure with an open top. The peg lacks pores or specialized socket structures [70]. In case of *Bombyx mori,* the hygrosensilla is entrenched within a grove with one side acting as a wall, shielding the hygrosensilla from its immediate environment [71] (Figure 2).

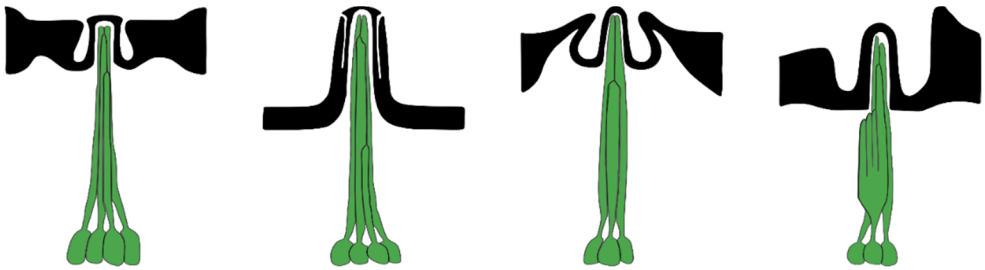

Figure 2: - Schematic illustrating hygrosensilla in different insects. From left to right- *Apis mellifera*, *Periplaneta americana*, *Carausius morosus* and *Bombyx mori.* Adapted from [71], with permission from Springer.

The hygrosensilla in honey bees, cockroaches, and stick insects differ in structure and position but share a common feature: they are not directly exposed to the immediate environment. Instead, they are shielded to varying degrees. In cockroaches, they are surrounded by towering sensilla such as trichoid and basiconic sensilla, while in honey bees and stick insects, they are protected by pit or wall-like structures. The positioning of hygrosensilla represents a balance between maximizing exposure and ensuring protection. Placing them distally on the antenna enhances their contact with ambient air, which is essential for accurately detecting changes in humidity. However, this location also exposes them to potential damage from environmental factors, such as physical abrasion or contamination from dust and debris. Given the relatively small number of these specialized sensilla, their protection becomes critical to maintain functionality.

Hygrosensilla houses three specialized HRNs arranged in a configuration known as the hygrosensory triad [9,71]. This triad works in unison to detect and measure changes in RH. It comprises a moist cell, which responds to increases in humidity; a dry cell, which detects decreases in humidity; and a hygrocool cell, which is sensitive to cooling. This precise arrangement allows insects to monitor their environmental conditions with remarkable accuracy, enabling vital behavioural and physiological responses to changes in humidity.

Hygrosensation in *Drosophila melanogaster*

Hygrosensory structures in *Drosophila melanogaster* antenna

Drosophila melanogaster, like other insects, possesses specialized structures for detecting humidity changes, which are located on the antenna within an invagination called the sacculus, situated on the posterior side of the third antennal segment (funiculus) (Figure 3). The sacculus is a multichambered pit organ approximately

55 µm long, oriented dorso-ventrally inside the funiculus and featuring a narrow 5 µm-wide opening on its medial surface. It consists of three chambers, each housing distinct types of sensilla with specialized structural and sensory roles (Figure 3).

Chamber I, about 15 µm deep, contains 5–7 no-pore sensilla basiconica (np-sb), which have an irregularly sculpted distal surface, lack a flexible socket at their base, and taper towards the distal end. Chamber II, approximately 13 µm in diameter, is divided into compartments by cuticular ridges, each housing a single no-pore sensilla coeloconica (np-sc). These np-sc sensilla resemble short, blunt-tipped pegs with smooth external surfaces and spongy internal cuticles. Chamber III, the largest chamber, measuring 25 µm in length and 15 µm in width, is divided into ventral and dorsal compartments by a cuticular flap. The ventral compartment contains thick, blunt-tipped grooved sensilla, while the dorsal compartment houses slender, pointed grooved sensilla [72].

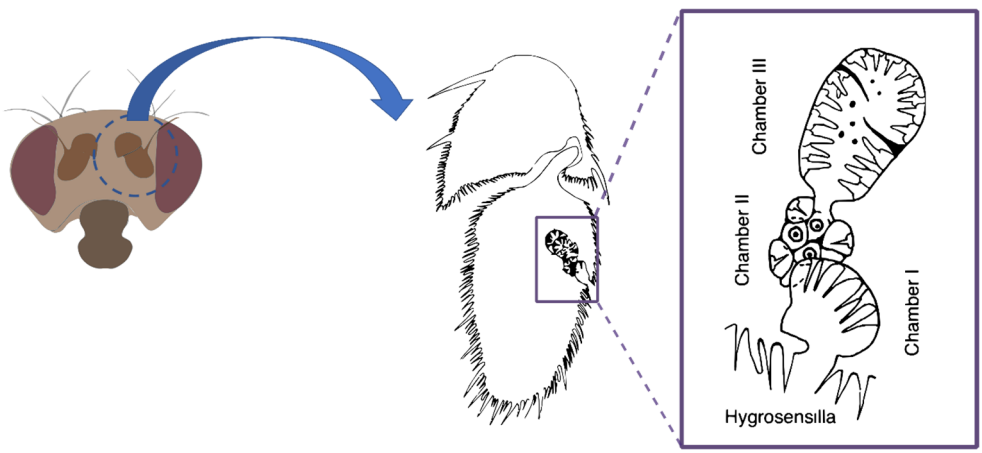

Figure 3: - Schematic Representation of the Sacculus in *D. melanogaster*. Illustration of the fly's head highlighting the position of the antenna (Left). The sacculus, a cavity located on the posterior side of the antenna, is strategically positioned to shield it from direct contact with the external environment. Center showing the sacculus's placement within the antenna and its internal structure (Right). The sacculus comprises three distinct chambers, each housing specialized sensilla. Adapted from [72] with permission from Springer.

Both np-sb and np-sc sensilla are considered hygrosensilla, meaning they are directly involved in humidity sensation. Characterized by their lack of pores, except for a molting pore at the distal tip of np-sc, these sensilla play distinct roles in the humidity sensation [72].

Hygrosensory receptor neurons

Due to the limited accessibility of the hygrosensilla in Drosophila, electrophysiological recording from individual hygrosensory neurons within the sacculus is challenging. This technical constraint has made it difficult to directly study their responses to environmental stimuli using single-sensillum electrophysiological techniques. Despite these challenges, it has been shown that the neurons housed within these sensilla function as detectors of humidity changes [73–76]. Each hygrosensillum contains a hygrosensory triad composed of three specialized neurons: one moist cell, one dry cell, and one hygrocool cell. Together, these neurons are primarily involved in sensing changes in humidity and temperature, as well as contributing to the sacculus's role in olfactory perception.

The HRNs express members of the large subfamily of ionotropic receptors [64]. They play a crucial role in humidity sensation in *D. melanogaster* through distinct moist and dry sensing pathways. Moist cells, which detect high humidity, express *IR25a*, *IR93a*, and *IR68a*. When humidity rises, these cells exhibit robust calcium responses. Conversely, dry cells, which respond to dry air, express *IR25a*, *IR93a*, and *IR40a* and shows opposite calcium responses to the given humidity stimulus when compared to the response of moist cells [74,76]. Both moist and dry cells contribute to hygrosensory behaviour, with IR-dependent pathways driving preference for dry environments in hydrated flies and moist environments in dehydrated flies. This behaviour suggests that the brain integrates information from these hygrosensory pathways with internal hydration signals to guide environmental preferences, ensuring adaptive responses to changing hydration states.

Proposed models for humidity sensing mechanism

The structure of hygrosensilla has been extensively studied using high-resolution imaging techniques, revealing detailed insights into their architecture. Additionally, the responses generated by the HRNs have been investigated through electrophysiology recordings and calcium imaging, providing valuable data on neuronal activity. Despite these advancements, a consensus on the exact mechanism underlying hygrosensation remains elusive. Currently, there are three widely accepted models that attempt to explain how humidity is sensed, namely the mechanical hygrometer model, psychrometer model and electrochemical hygrometer model [71].

Mechanical Hygrometer Model

The Mechanical Hygrometer Model explains hygrosensation in insects as a process where the hygrosensillum functions as a hydromechanical transducer, with the cuticular wall acting as the primary sensor. The cuticle deforms in response to

changes in humidity, swelling when absorbing water at high humidity and shrinking in dry conditions. These mechanical changes are directly transmitted to the dendritic membranes of HRNs, where they generate neuronal responses through deformation-induced voltage changes. Applying a mechanical force to the cuticular wall of hygrosensilla, such as by increasing air pressure in insects like *P. americana* and *Carausius morosus* has shown to alter neuronal firing rates, indicating that direct mechanical force impacts sensory responses [77].

For effective transduction in the mechanical hygrometer model, there needs to be a close association between the inner cuticle wall and the dendritic membranes of the HRNs. However, the presence of lymph in this region could create a cushioning effect, dampening the effect of mechanical deformations thereby reducing neuronal sensitivity. Another limitation of this model lies in the hygroscopic nature of the cuticle; without proper isolation along the inner wall, it risks absorbing water from the lymph inside the hygrosensillum rather than solely responding to external humidity changes.

Psychrometer Model

The psychrometer model proposes that HRNs function as thermoreceptors rather than mechanoreceptors, using evaporation-induced cooling to assess RH. In this mechanism, evaporation from a "wet" surface on the sensillum causes cooling, while a "dry" surface remains unaffected. The temperature difference between these surfaces indicates humidity levels: drier air intensifies evaporation, resulting in greater cooling and a larger temperature depression. The model relies on two thermoreceptive cells—one linked to the wet surface and the other to the dry surface—where the temperature difference between them serves as a measure of RH.

Even though the model presents a valid explanation, it still presents significant challenges. Maintaining a wet surface on the cuticle requires a constant and precisely regulated water supply. Proximity between the wet and dry surfaces introduces another issue, as evaporative cooling from the wet surface can influence the dry surface, reducing the temperature differential and potentially underestimating humidity. Furthermore, species such as *P. americana* display a dual response in their dry cells, which react to both humidity changes and cooling. This duality suggests the involvement of an additional mechanism, such as humidity-induced swelling or shrinkage, that complements evaporative cooling.

Electrochemical Hygrometer Model

The electrochemical hygrometer model provides a unique framework for understanding hygrosensation by emphasizing the role of electrolyte concentration

changes within the lymph surrounding the dendrites of HRNs. According to this model, ambient humidity influences the ionic composition of the lymph surrounding the dendrites. As humidity decreases, increased evaporation from the lymph raises the electrolyte concentration, which in turn alters the ionic potential across the dendritic membranes, triggering changes in sensory responses.

However, the model faces notable challenges, particularly in regulating lymph flow. If the flow is too slow, excessive electrolyte accumulation under dry conditions can disrupt neuronal function. Conversely, if the flow is too fast, it may fail to accurately reflect changes in humidity, diminishing sensitivity. Additionally, achieving consistent evaporation rates and maintaining adequate lymph reservoirs to support sustained ionic regulation remain critical but unresolved aspects of the model.

Challenges to existing models for the mechanism of hygrosensation

Electrophysiological studies challenge all three hygrosensory models by revealing contradictions in their assumptions about humidity sensing. The mechanical hygrometer model bases itself on the humidity-induced shrinking or swelling of the sensillum's cuticular wall deforming dendrites and triggering a neuronal response. However, atomic force microscopy scans of honeybee hygroreceptive sensilla showed no changes in cuticular wall dimensions across a range of humidity levels [78]. Additionally, the temperature-dependent responses of cockroach moist and dry cells to RH undermine the model’s premise that structural deformation is solely dictated by RH [79]. The psychrometer model, which relies on evaporative cooling to measure humidity, also falls short. In cockroach dry cells, respond to both lowering of RH and to cooling. This suggests that the dry cell's response to these two stimuli is not straightforward and cannot be explained by psychrometer model alone [80]. The electrochemical hygrometer model, which attributes sensory responses to humidity-induced changes in lymph electrolyte concentration, similarly fails to account for the observed interplay between temperature and humidity in HRN responses. Together, these findings suggest that no single model fully explains hygrosensation, pointing instead to a multimodal mechanism integrating mechanical, osmotic, and thermal cues.

Although it is theoretically possible for a single indirect stimulus to drive hygrosensation, the organization of the hygrosensory triad —made up of temperature-sensing and humidity-sensing neurons—suggests that multiple independent stimuli are involved. For instance, research on *C. elegans* has shown that both mechanosensory channels (MEC-6/MEC-10/ASIC-1) and thermosensation-related channels (TAX-4) play a role in learned hygrotaxis [81]. This indicates that insects, too, may rely on more than one sensory pathway to detect humidity.

The presence of hygrocool cells and the common use of evaporative cooling in the physiology of animals from insects to mammals, further highlight the importance of evaporation in humidity sensing. According to Tichy et al, moist cells become active when evaporation rates decrease, while dry cells respond to increasing evaporation rates [79]. These changes in evaporation could be detected through various mechanisms, such as shifts in lymph concentration (osmosensation), pressure changes from osmotic forces (shear stress), mechanical stress on dendrites, or changes in temperature caused by evaporation (thermosensation).

Although HRNs likely respond to evaporation rates, it is still unclear whether they detect these rates as mechanical, osmotic, or thermal signals—or a combination of all three. Current evidence suggests that insects use a multimodal system, combining multiple sensory inputs to accurately respond to environmental humidity changes.

Aims

The primary aim of this thesis is to advance our understanding of hygrosensation, using *D. melanogaster* as a model system. Despite progress in identifying genes and structural features associated with this sensory modality, the underlying transduction mechanisms of hygrosensation remain poorly understood. To address this gap, this research investigates behavioural responses to humidity stimuli, identifies key genes involved in this sensory process along with examining structural differences of hygrosensory organs under high and low humidity conditions. By integrating these approaches, the thesis aims to uncover the molecular and structural basis of humidity sensing and its role in guiding insect behaviour.

The following studies were conducted to achieve these objectives:

Paper I: A dynamic humidity arena to explore humidity-related behaviours in insects

The objective was to develop a humidity arena capable of exposing flies to a continuous humidity range of 10–80% RH, enabling the study of quantifiable behavioural responses to humidity changes.

Paper II: Conserved molecular signatures of hygrosensory neurons in two dipteran species

The aim was to identify the molecular players involved in hygrosensory transduction by integrating comparative transcriptomics with targeted behavioural analyses.

Paper III: Humidity-dependent structural adaptations of *Drosophila melanogaster* hygrosensilla

This study aimed to investigate the mechanical hygrometer model to determine if there are any dimensional variations in hygrosensilla between two fly samples exposed to high and low humidity environments.

Materials and methods

The current thesis describes three studies investigating different aspects of humidity sensing in *D. melanogaster*. The research employs a comprehensive approach, utilizing diverse methods detailed below. Only the methods designed and implemented by the author of this thesis have been presented

Model organism

D. melanogaster was chosen as the model organism for this study due to its extensive genetic toolkit and its established use in studying humidity-dependent behaviours. Its well-characterized genome and the availability of transgenic techniques, such as GAL4/UAS driver systems [82] and RNA interference (RNAi) [83,84], allow precise manipulation and analysis of specific genes. Additionally, mutant fly lines targeting key ionotropic receptors (IRs) implicated in humidity detection provide a powerful resource to dissect the molecular and neuronal mechanisms underlying humidity sensing and related behaviours. These features make Drosophila an ideal model for advancing our understanding of hygrosensory pathways.

Fly handling and Fly lines

Flies were reared in vials containing cornmeal agar medium maintained at 25° C under 12-hour dark/light cycle within an incubator. Inside home vials, humidity levels ranged from 90% RH near the food to 70 % RH farthest away from the food. For experiments, eclosed flies were collected and acclimated to room temperature conditions (21-23° C) in home vials. The flies utilized in the experiments performed were approximately 7-14 days old.

Different fly strains were used across the experiments described in Papers I, II, and III. Stocks were obtained from the Bloomington Drosophila Stock Center (BDSC). In Paper I, w^{1118} (Bloomington ID: 5905) was used as a control, while $Ir93a^{MI05555}$ (Bloomington ID: 42090), a strain deficient in humidity sensing, served as the humidity-blind group. Paper II employed w^{1118} to study the structure of the sacculus and hygrosensilla. For the behavioural response experiments in Paper II, apart from w^{1118}, several other genetic lines were utilized, including $5\text{-}HT7^{attp}$ (Bloomington ID: 84446), nub^{2} (Bloomington ID: 358), $Kif19A^{MI12222}$ (Bloomington ID: 56735), and $Ir21a^{EP526}$ (Bloomington ID: 17177) to study humidity-induced behavioural

changes. Both male and female flies were used for experiments in Paper I and III. Only female w^{1118} flies were used in Paper III.

Sample preparation

Tethering of flies

To tether flies, selected individuals were anesthetized on a chilled metal plate. Once immobilized, the flies were positioned upright on their legs, exposing the thorax. A pin coated with a light-curing adhesive (HelioBond) was carefully affixed to the thorax, and the adhesive was cured by shining a blue LED light (430–450 nm, 63 µW mm², Radii-cal, SDI Ltd, Bayswater, VIC, Australia) for 5–6 seconds.

Desiccation

Desiccation of flies was performed in a custom-designed desiccation chamber, constructed from a cylindrical container equipped with an inlet for dry air to maintain controlled humidity conditions. The chamber's top was covered with a sponge, which partially restricted airflow to reduce turbulence within the chamber while still allowing a steady flow of dry air. The flies, tethered to pins, were carefully attached to the sponge so that their bodies were positioned inside the chamber. Once positioned, the sponge was securely placed, enclosing the flies within the desiccation environment. The RH within the chamber was maintained around 8–10% RH, ensuring consistent conditions for desiccation. Flies used in the behaviour experiments were desiccated for 4 hours.

Plunge Freezing

Flies were preserved under specific humidity conditions using the method of plunge freezing. To prepare the samples, flies were attached to the tip of a forceps and positioned within the humidity- and temperature-controlled chamber of a Vitrobot Mark IV system (FEI, Denmark). They were maintained in the chamber at the desired humidity level (either 26% RH or 80% RH) and a temperature of 22°C for approximately 45 seconds. Following this acclimatization, the flies were rapidly plunged into a crucible containing liquid ethane, which was itself surrounded by liquid nitrogen. The use of liquid ethane instead of liquid nitrogen for plunge freezing is critical due to their differing thermal properties. While liquid nitrogen has a very low temperature (approximately -195°C), its low heat capacity causes it to boil off rapidly when a room-temperature sample is introduced, slowing the freezing process of the sample and allowing the formation of ice. In contrast, liquid

ethane, which has a much higher heat capacity and remains liquid at temperatures slightly above those of liquid nitrogen (melting point: -188°C), ensures rapid and uniform freezing of the sample. This minimizes ice crystal formation and better preserves the fine structural details of the flies at the desired humidity level.

Freeze substitution and Staining

The staining and preparation of samples for electron microscopy involved a detailed freeze substitution, staining, and dehydration protocol adapted from [85]. After plunge freezing, samples were subjected to a freeze substitution protocol, a method that gradually replaced water in the frozen tissue with organic solvents at low temperatures [86]. This method ensures that structural integrity is maintained by preventing ice crystal formation. In this study, plunge-frozen samples were transferred to a substitution medium containing 0.1% tannic acid and 0.5% glutaraldehyde in acetone and incubated at -90°C for 96 hours. Tannic acid acts as a fixative and enhances contrast by binding to specific cellular components, while glutaraldehyde is a cross-linking agent that stabilizes proteins and preserves structural integrity [87–89].

After incubation, the substitution medium was removed through four washes with anhydrous acetone, and the samples were then exposed to 2% osmium tetroxide (OsO_4) in anhydrous acetone for 28 hours. Osmium tetroxide is a heavy metal fixative that reacts with lipids, imparting electron density and contrast to membranes and other lipid-rich structures. This step also further preserves the morphology of cellular components and adds contrast [90,91].

The temperature was gradually increased over 14 hours to -30°C, and the samples were held at this temperature for an additional 16 hours to allow for controlled chemical interactions. Residual OsO_4 and substitution medium were removed with four more washes in anhydrous acetone before the temperature was further raised to 1°C over four hours. The sample was further dehydrated using a series of ethanol and acetone solutions followed by embedding with epoxy resin to facilitate trimming and sectioning for imaging

Data collection

Dynamic humidity arena

Binary choice assay was the primary technique used to establish the humidity preference in *D. melanogaster* [74,75,92,93]. While this method provided a straightforward approach to assess preference between two distinct humidity levels, it had several limitations. Binary choice assays often lacked the ability to mimic

natural humidity gradients, restricted the exploration of continuous humidity preferences, and could oversimplify the complexity of insect hygrosensory behaviour. To address these shortcomings, the dynamic humidity arena was developed. This arena allows the generation of continuous or stepwise humidity gradients (e.g., 10-80% RH), enabling more ecologically relevant experiments. By providing fine environmental control and facilitating quantifiable behavioural analysis, the dynamic humidity arena offers a more comprehensive understanding of humidity-driven behaviour, surpassing the constraints of binary choice assays. The behaviour data used in Paper I and Paper II were acquired by utilizing this arena.

The dynamic humidity arena was developed as part of this thesis to address limitations in existing methods for studying insect hygrosensation. The system integrates a fly-on-ball setup with real-time humidity control [79,94], offering an improved platform for investigating precise behavioural responses to continuous humidity gradients. The design combines established tracking techniques with a custom-built stimulus delivery system, providing an original and reproducible approach for examining humidity-dependent behaviour in *D. melanogaster*.

A proportional-integral-derivative (PID) control algorithm was implemented to dynamically regulate humidity delivery. This algorithm operates by continuously calculating the difference, or error $e(t)$, between the measured and desired RH and adjusting the mixing of dry and moist air streams through proportional valves. The PID control output $\mu(t)$ is computed using three gain parameters: proportional K_p, integral K_i, and derivative K_d which collectively ensure stability, accuracy, and responsiveness of stimulus delivery.

$$\mu(t) = K_p\, e(t) + K_i \int_0^t e(\tau) d(\tau) + K_d \frac{d\, e(t)}{dt}$$

Feedback from a digital humidity sensor enables real-time recalibration of the airflows every 0.5 seconds, allowing the system to maintain a smooth and stable humidity gradient (10–80% RH) tailored to experimental conditions. This precise regulation of humidity stimuli ensured consistency across trials, facilitating detailed behavioural analysis (Figure 4).

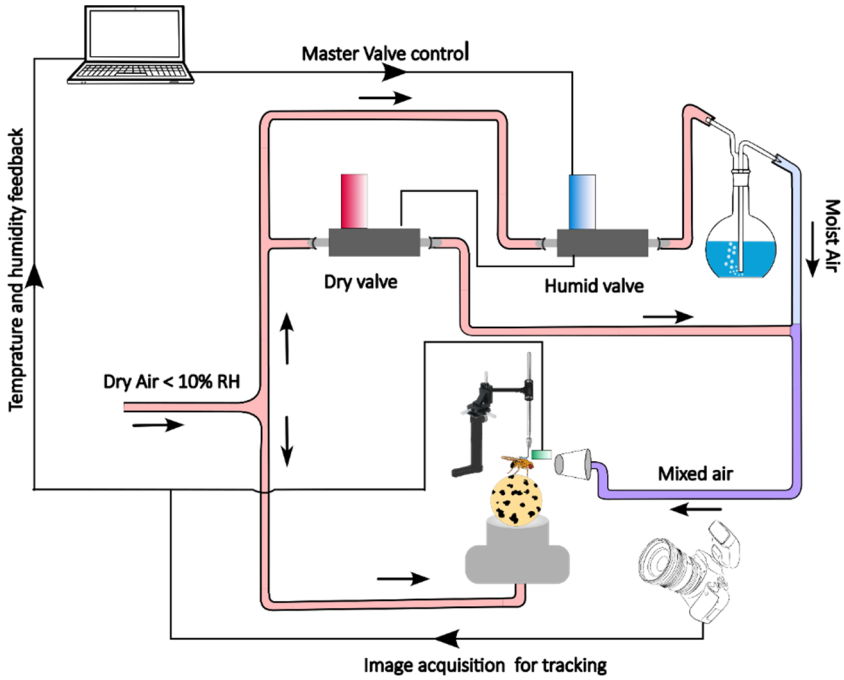

Figure 4: - Schematic illustration of the dynamic humidity arena. The dry and moist air are mixed in required proportions by regulating their flow using the dry valve and humid valve. A humidity and temperature sensor shown in green placed above the fly's head close to the antenna sends the feedback response to the computer that employs a proportional-integral-derivative (PID) control algorithm to optimise the output of the flow meters to minimise the error in the humidity set points. A high-speed USB camera acquires the rotation of the ball to calculate the trajectory of the tethered fly. Using the calculated trajectory, RH values are adjusted depending upon the experimental protocol

The tethered fly controlled the rotation of a suspended ball through its walking behaviour, with the movement data captured in real time using FicTrac software [95]. This approach enabled accurate tracking of the fly's response to varying humidity levels.

The dynamic humidity arena can operate in two distinct modes, each designed to examine specific aspects of humidity-dependent behaviour in flies.

In the dynamic mode, humidity levels are dynamically adjusted in real time based on the fly's fictive position within the arena. This is achieved using a triangular wave pattern as the humidity map, where the humidity is linearly scaled relative to the fly's fictive position. The map features two configurations: a low-to-high gradient, where humidity increases radially and then decreases (Figure 5 A), and a high-to-low gradient, where humidity decreases radially and then increases (Figure 5 B), both maintaining a constant slope. Importantly, this humidity map is indefinite, meaning that the cycle of humidity changes continuously repeats, unbound by

distance. As a result, the fly can move freely throughout the arena without disrupting the set stimulus map, ensuring consistent exposure to the humidity gradient. This setup enables the tethered fly to explore a continuous humidity gradient ranging from 10% to 80% RH, allowing it to locate and remain within a humidity range that best suits its physiological needs. This allows the determination of desired humidity levels or humidity preference in the tested flies

The second mode, referred to as the forced humidity mode, exposes the fly to predetermined humidity levels for a fixed duration. This paradigm is particularly useful for observing behavioural changes that occur when the fly is subjected to sudden shifts in humidity (Figure 5 C). Due to the rapid and noticeable behavioural responses triggered by these transitions, this mode becomes an ideal approach for screening flies with genetic mutation to identify genes involved in humidity sensation

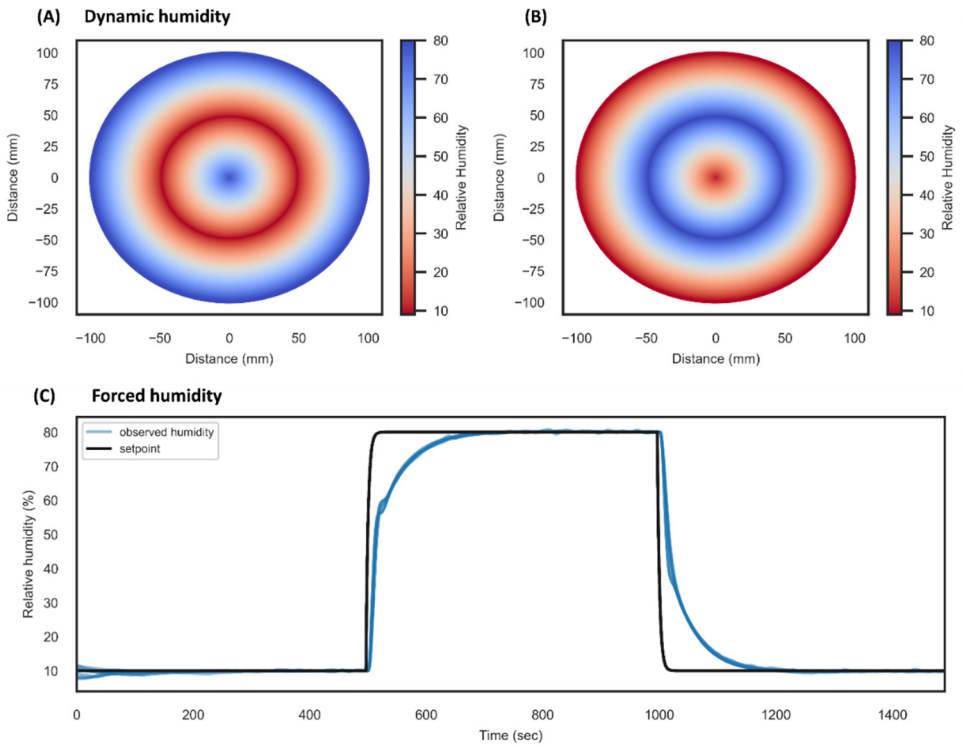

Figure 5: - Humidity stimulus protocols. Dynamic humidity mode, showing the schematic diagram of high-to-low (A) and low-to-high (B) humidity maps, here humidity is dependent on the radial distance from the centre. (C)Traces showing set-point and measured humidity over time for the forced humidity protocol.

To streamline experiments and data acquisition, a graphical user interface (GUI) was developed using Python's Qt Designer module. The GUI provided features to initiate and stop experiments, manage experimental parameters, and store recorded data. Key parameters, including relative positional coordinates, delivered humidity, and temperature, were also logged in real time and saved through the interface for subsequent analysis.

While the dynamic humidity arena offers precise control and versatility, it does have some limitations. One key constraint is the lag observed when changing RH around the fly, requiring approximately 81 seconds to reach 90% of the set point regardless of the magnitude of the change. This delay is primarily due to the low flow rate of 1 l min^{-1} used to deliver the stimulus. While higher flow rates could reduce this lag, they would introduce undesired head-on wind effects that might confound the behavioural responses. A more aggressive tuning of the PID parameter could also shorten the latency in settling time but will also introduce additional overshoot of RH from the set point, which will result in multiple oscillations around the target value. These oscillations would expose the fly to repeated small multi-directional changes of RH, potentially causing undesirable behavioural responses.

Serial block face scanning electron microscopy

Serial block-face scanning electron microscopy (SBF-SEM) is a high-resolution imaging technique that allows for three-dimensional reconstruction of biological structures [96–98]. In this method, the surface of a resin-embedded sample is sequentially imaged with an electron beam, and then ultrathin sections are removed using an in-chamber microtome, revealing new surfaces for subsequent imaging. This iterative process produces a series of detailed images that can be computationally reconstructed to visualize internal structures in three dimensions with nanometer-scale resolution. Once imaging parameters are fixed, the process is fully automated, enabling the imaging of large samples efficiently. This made SBF-SEM an ideal method for capturing high-resolution images of the entire volume of the Drosophila antenna, essential for studying the structural adaptations of hygrosensilla.

To acquire high-resolution images of antennal sections, the embedded resin block was carefully positioned and trimmed to achieve an optimal angle for imaging. Serial block-face scanning electron microscopy (SBF-SEM) was performed using the Teneo VolumeScope (FEI, Denmark) (Figure 6).

For the high humidity (80% RH) dataset, two sets of images were collected. In the first set, imaging covered a field of view of 42.5 µm × 42.5 µm, with a total depth of 14.46 µm, using a resolution of 10.38 nm × 10.38 nm in the XY plane and 30 nm in the Z-axis. A total of 482 sections were acquired at 30 nm thickness per slice, with beam settings of 2.28 keV energy, a beam current of 400 pA, and a dwell time

of 3 µs. Multi-Energy Deconvolution (MED) was applied to enhance contrast and reduce noise. Following an interruption, imaging resumed with updated parameters, expanding the field of view to 60.0 µm × 60.0 µm and a depth of 30.03 µm, with a resolution of 14.65 nm × 14.65 nm in the XY plane and 30 nm in the Z-axis. This second set included 1,001 slices, captured under similar beam conditions but with an electron dose adjustment.

For the low humidity (26% RH) dataset, uninterrupted imaging was conducted across a larger field of view (111.0 µm × 111.0 µm) and depth (104.16 µm), with a resolution of 10.84 nm × 10.84 nm in the XY plane and 30 nm in the Z-axis. A total of 3,472 sections were collected with beam settings of 2.27 keV energy, a dwell time of 1 µs, and an electron dose of 10.62 e/nm². In all datasets, imaging was performed under low vacuum conditions, using a VsGAD detector and consistent integration settings.

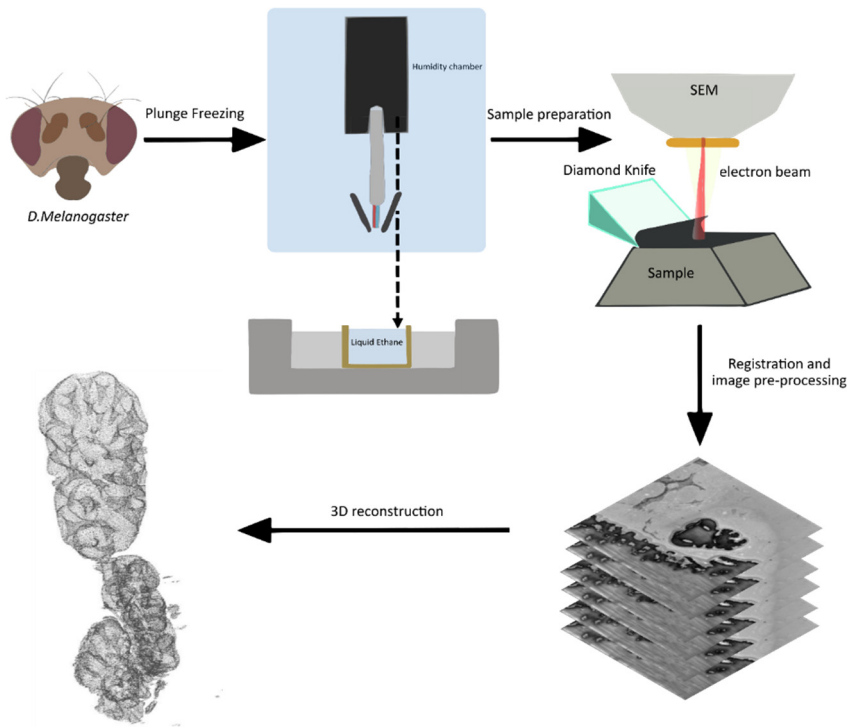

Figure 6: - Workflow for Sample Preparation, Imaging, and Segmentation of *D. melanogaster* Sacculus under specific Humidity Conditions. Schematic representation of the workflow: Flies were acclimated in a humidity-controlled chamber (26% or 80% RH) for 30 seconds before being plunge-frozen in liquid ethane to preserve their structural integrity. Samples were subsequently stained, embedded in epoxy resin, and trimmed to expose the antenna for imaging. High-resolution imaging of the entire antenna was performed using serial block-face scanning electron microscopy (SBF-SEM). A U-Net model was employed for segmentation of the sacculus from the acquired images, enabling 3D reconstruction based on the segmented contours.

Data processing and analysis

Behavioural data analysis

Behavioural data obtained from the dynamic humidity arena was processed to extract meaningful insights into fly responses under different experimental conditions. The raw data, stored as CSV files, was imported into Python for analysis using the Pandas library [99,100]. To reduce noise and enable smoother analysis, an exponentially weighted moving average filter, with a center of mass of 50, was applied to the positional coordinate data.

Using the filtered x and y positional coordinates, the Euclidean distance between consecutive data points was calculated. Cumulative distance was then derived by summing these Euclidean distances over time, allowing for the computation of walking speed (hereafter referred to as "speed") based on distance covered within each time interval recorded during the experiments.

For humidity preference analysis, histograms of RH distribution were constructed using bins of 5% RH, corresponding to the humidity map employed in the experiments. Individual preferences within each experimental group were further examined by extracting the preferred RH for each fly and summarizing the results in boxplots.

In experiments comparing fly speeds at forced humidity levels (10% and 80% RH), the recorded speeds were normalized to their respective peak speeds to account for variability among individuals. Normalized speeds were averaged over 1-second windows, enabling comparison both within and between experimental groups under specific time conditions.

Image scaling and alignment

During the imaging of the high-humidity sample, an interruption necessitated repositioning the sample and adjusting the imaging parameters. This led to differences in field of view, pixel resolution, and scaling between the first set of images (acquired before the interruption) and the second set (acquired after the interruption) (Figure 7 A). To ensure consistency in the final dataset, rescaling and alignment of the two image sets were performed.

For rescaling, the pixel dimensions of the second set were adjusted to match the resolution of the first set. This was achieved by determining the scaling factor based on the known differences in pixel size between the two datasets. The images in the second set were resized accordingly, ensuring that the physical dimensions of structures were uniformly represented across both sets.

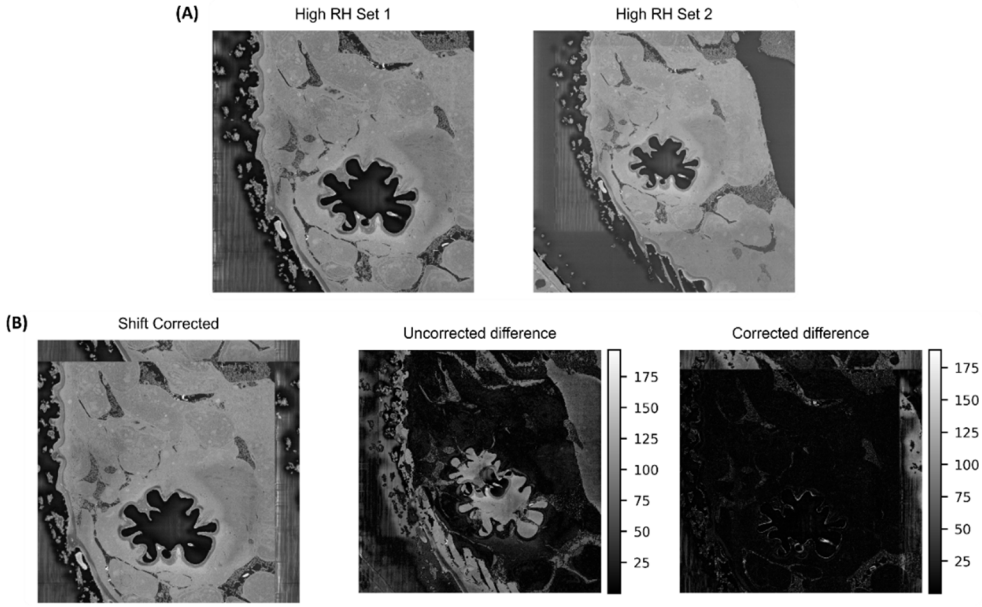

Figure 7: - Image scaling and alignment (A) High humidity sample imaged before (Left) and after interruption during image acquisition, leading to two sets of images with different resolution. (B) High RH sample images (Set 2) adjusted to match Set 1 resolution (Left), with difference images showing Set 1 vs. Set 2 before (Centre) and after (Right) shift and resolution correction.

After scaling, the relative shift between the two set was identified using phase cross-corelation in Fourier space by using phase_cross_corrrelation function from scikit-image library, which also employed upsampled matrix-multiplication DFT to achieve arbitrary subpixel precision [101]. The calculated shift was then applied to the rest of the images to align them with the reference image. This created a seamless overlap between the two datasets, ensuring that features in both sets aligned correctly in the merged 3D reconstruction (Figure 7 B).

Image segmentation

To segment the sacculus from the acquired images, a deep learning-based approach was implemented using a U-Net model [102]. Labels for training were generated by manually tracing the sacculus using IMOD software, which created binary masks representing the region of interest [103]. To enhance the diversity of the training dataset, image augmentation techniques were applied. Each image was flipped vertically and horizontally and rotated by 45 and 130 degrees, effectively increasing the number of training samples. The images and corresponding binary masks were downsampled to 256 × 256 pixels and normalized to scale pixel values between 0 and 1. The dataset was then split into training, validation, and test sets in a 90:5:5 ratio.

The U-Net model was trained using the Adam optimizer with binary cross-entropy as the loss function. Two evaluation metrics—accuracy and Intersection over Union (IoU)—were employed to assess the model's performance. Given that the sacculus occupies a smaller area compared to the background, greater emphasis was placed on the IoU metric, as it measures the overlap between the predicted mask and the ground truth more robustly. Two TensorFlow callback functions were used to optimize the training process. The ModelCheckpoint callback saved the model with the best IoU score during training, ensuring the retention of the most accurate model. The ReduceLROnPlateau callback dynamically reduced the learning rate whenever validation loss plateaued, promoting better convergence [104]. Using the trained model, segmentation predictions were generated for both the low and high-humidity datasets.

Orientation and measurement of dimensions

To facilitate dimensional measurements of the hygrosensilla, the contours of the segmented images were extracted using the OpenCV library [105]. Stacking these contours provided a three-dimensional (3D) representation of the sacculus, which was subsequently converted into point clouds for visualization and dimensional analysis. Individual sensilla were cropped from the point cloud for further processing. However, due to the random orientation of the hygrosensilla within the sacculus, standardizing their alignment was necessary to streamline automated dimensional measurements.

For sensilla in Chambers I and III, a representative sensillum was selected and its point cloud was centered at the origin by subtracting its centroid coordinates from all points. Principal Component Analysis (PCA) was applied to this centered point cloud to determine its primary axes. In these chambers, the axis with the greatest variance corresponded to the sensillum's length, which was designated as the principal axis. The angle between the principal axis and the Z-axis was calculated, and the representative sensillum was rotated to align its principal axis with the Z-axis, ensuring it was oriented in the required direction.

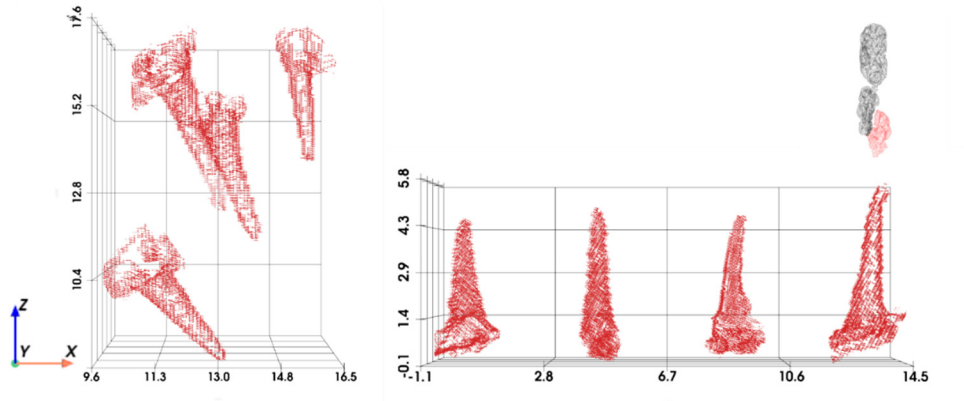

Figure 8: - Alignment of sensilla. Isolated sensilla from Chamber I, shown in their original orientation within the sacculus (Left) and after alignment for dimensional measurement (Right).

Once the representative sensillum was aligned, it served as the reference for orienting the remaining sensilla in the same chamber. A RANSAC-based global registration algorithm was used for this step [106]. The remaining sensilla point clouds were downsampled to enhance computational efficiency, and the relative shift and rotation needed to align each sensillum to the already-aligned representative sensillum were determined. A transformation matrix, computed by the RANSAC algorithm, was then applied to each point cloud to ensure precise alignment of all sensilla within the chamber to the reference orientation (Figure 8).

In Chamber II, where the sensilla's length and width were similar, PCA could not reliably determine the principal axis. Instead, a synthetic conical point cloud resembling the shape and dimensions of Chamber II sensilla was created and oriented along the Z-axis. This synthetic model served as the alignment target. The sensilla in Chamber II were aligned to this target using a RANSAC-based registration process similar to that used in the other chambers (Figure 9).

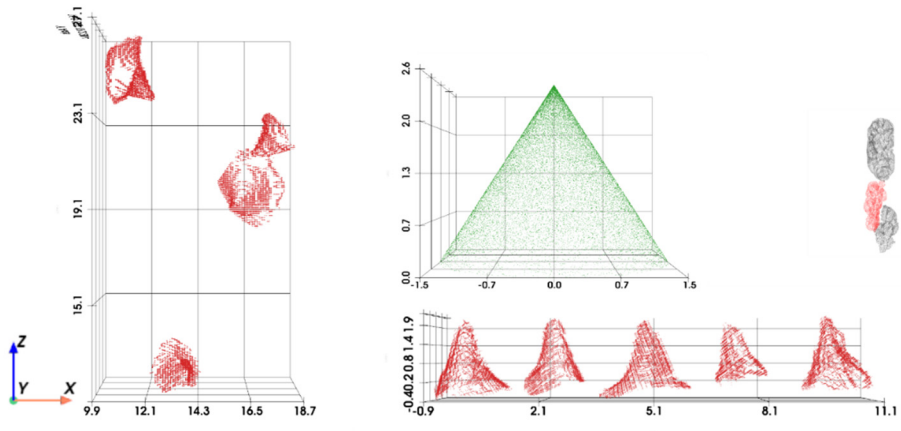

Figure 9: - Alignment of sensilla. Isolated sensilla from Chamber II, shown in their original orientation within the sacculus (Left) and after alignment for dimensional measurement (Right). The green point cloud was used to as a reference target for the alignment of the sensilla in Chamber II

Once aligned, the point clouds were converted into 3D meshes using Delaunay triangulation. These meshes were then sliced along the Z-axis to generate two-dimensional (2D) cross-sectional slices at different heights. For each slice, the maximum distance between extreme points in the XY plane was measured to determine the width at that height. By repeating this process across the height of the sensillum, a detailed width profile was constructed.

To minimize noise in the measurements, a weighted moving average (WMA) filter was applied to the width profile, smoothing out any irregularities. The final smoothed data was plotted to reveal the width distribution along the length of the hygrosensilla

Statistical analysis

The statistical analysis for Paper I was conducted using the SciPy and Statsmodels libraries in Python [107,108]. A Mann–Whitney U-test with Bonferroni correction was employed for both humidity preference analysis and comparisons of walking speed in the forced humidity experiments. This non-parametric test was chosen because it does not assume normality, making it well-suited for data with non-normal distributions. Additionally, the Mann–Whitney U-test is robust for comparing distributions between independent groups, even with small sample sizes [109]. The Bonferroni correction was applied to account for multiple comparisons, minimizing the risk of Type I error [110].

To model the relationship between humidity and fly speed in, a mixed-effects model was implemented using the Statsmodels library in Python [108,111]. This method incorporated fixed effects, including humidity, group, sex, and their interactions, to capture the average influence of these factors on speed. Random effects were included for individual identifiers to account for baseline variability in walking speeds, improving the model's ability to reflect both overall trends and individual differences. To meet the normality assumption required for the model, a Box–Cox transformation was applied to the speed data using the SciPy library, followed by an inverse transformation to obtain adjusted speed profiles for various sex and group combinations at specific humidity levels.

In Paper II, statistical analysis was performed using a custom written Python script that utilized the NumPy library to conduct a non-parametric bootstrap test with 10,000 resampling iterations [99,112]. This method was chosen due to the non-normal distribution of the data. The observed difference in mean walking speed between humidity levels was calculated, and speed values across the humidity stimuli were pooled and resampled with replacement to generate new datasets. For each resample, the difference in means was computed to form a distribution of bootstrap differences. The p-value was determined by calculating the proportion of bootstrap differences greater than or equal to the observed difference in absolute value. Comparisons were made between the three RH setpoints: initial 10% RH, 80% RH, and final 10% RH.

For Paper III, statistical analysis was conducted to examine the relationship between sensilla width and height under varying humidity conditions. A mixed-effects model from the Statsmodels library in Python was employed to analyze this relationship, with a fixed effect for the interaction between height and group (humid vs. dry) to capture the influence of environmental conditions on width. A random effect for individual sensilla was incorporated to account for inherent variability between sensilla, ensuring the model adjusted for baseline differences and focused on the impact of humidity conditions rather than natural variation. Model predictions for sensilla width across a range of heights were generated for both groups, and 95% confidence intervals were calculated to evaluate the uncertainty in these estimates.

To provide a standardized metric for comparing the overall size of sensilla, their dimensions were further assessed using the full width at half maximum (FWHM). Differences in FWHM distributions between groups were evaluated using a non-parametric Mann–Whitney U-test.

Results and discussion

Paper I: - A dynamic humidity arena to explore humidity-related behaviours in insects

The study aimed to develop a novel experimental arena for investigating humidity-guided behaviour in *D. melanogaster* across a continuous humidity range of 10–80% RH. The arena facilitated the examination of humidity preferences and the impact of internal states on humidity-dependent behaviours. A summary of the findings is presented below.

The preference towards a specific humidity range was assessed using the dynamic mode for the experiments performed in the dynamic humidity arena, which allowed the tethered fly to sample all humidity values between the range of 10 and 80 % RH. The RH was adjusted automatically based on the trajectory of each fly within a preset humidity map, either transitioning from a low to high or high to low RH.

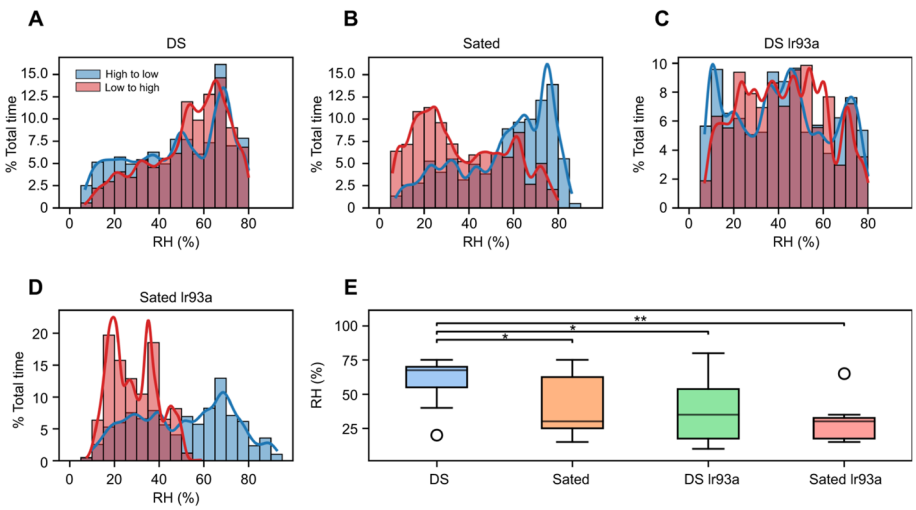

Figure 10: - RH preference in dynamic humidity arena. (A–D) Collective humidity distribution for trials within the desiccated and starved (DS; n=9), Sated (n=12), DS Ir93a (n=10) and Sated Ir93a (n=7) groups. The peaks in the histogram plot represent the humidity range in which the flies spent the maximum time during the observation period. The two different colours in the histogram represent the type of humidity map used during the experiment (high to low and low to high). (E) Preferred humidity based on time spent in a given humidity range for individual trials. Box plots show the median, upper and lower quartiles and 1.5× the interquartile range; circles represent outliers. Asterisks indicate statistical significance between the DS group and the remaining groups (Mann–Whitney U-test with Bonferroni correction: *P<0.05, **P<0.01).

Desiccated w^{1118} flies (DS) consistently exhibited a preference for RH levels between 65% and 70%, regardless of the humidity map used. In contrast, Sated flies displayed no specific humidity preference and tended to remain within the initial RH environment they were introduced in. Flies exposed to a low-to-high humidity map stayed within an initial range of 15–25% RH, whereas those exposed to a high-to-low humidity map remained within an initial range of 75–80% RH (Figure 10 A-B).

The humidity impaired DS Ir93a group demonstrated a much broader humidity distribution, indicating that these flies did not settle at a specific RH. Meanwhile, Sated Ir93a flies behaved similarly to the Sated group, remaining within their initial RH range depending on the humidity map employed (Figure 10 C-D). Statistical analysis revealed significant differences in the distribution of humidity preferences between the DS group and all other groups ($P < 0.05$) (Figure 10 E).

These findings indicate that flies capable of sensing changes in RH, when desiccated and starved, exhibit a strong preference for an RH level between 65% and 70%. However, when fully sated, humidity is not a critical factor, as flies in both the Sated and Sated Ir93a groups remained within their starting RH range regardless of the humidity map used during the experiment. This suggests that the desiccation and starvation drives a specific humidity preference, while sated flies, regardless of their sensory capacity, do not prioritize humidity

The behavioural response of flies to sudden shifts in humidity was assessed using a forced humidity protocol. In this setup, flies were sequentially exposed to humidity levels of 10% and 80% RH, with each level maintained for 500 seconds. The walking speed of the flies was measured at each humidity level to quantify their response to these abrupt changes.

To validate the speed–humidity relationship observed in the dynamic humidity arena, we used the dynamic humidity arena in the forced humidity mode. DS flies exhibited a significant reduction in walking speed when transitioning from 10% to 80% RH ($P<0.01$). This response was distinct to DS flies, as no comparable changes were observed in the Sated, DS Ir93a, or Sated Ir93a groups. For these groups, the speed distributions remained consistent across the two humidity levels, and no statistically significant differences were detected ($P>0.05$) (Figure 11 A-D).

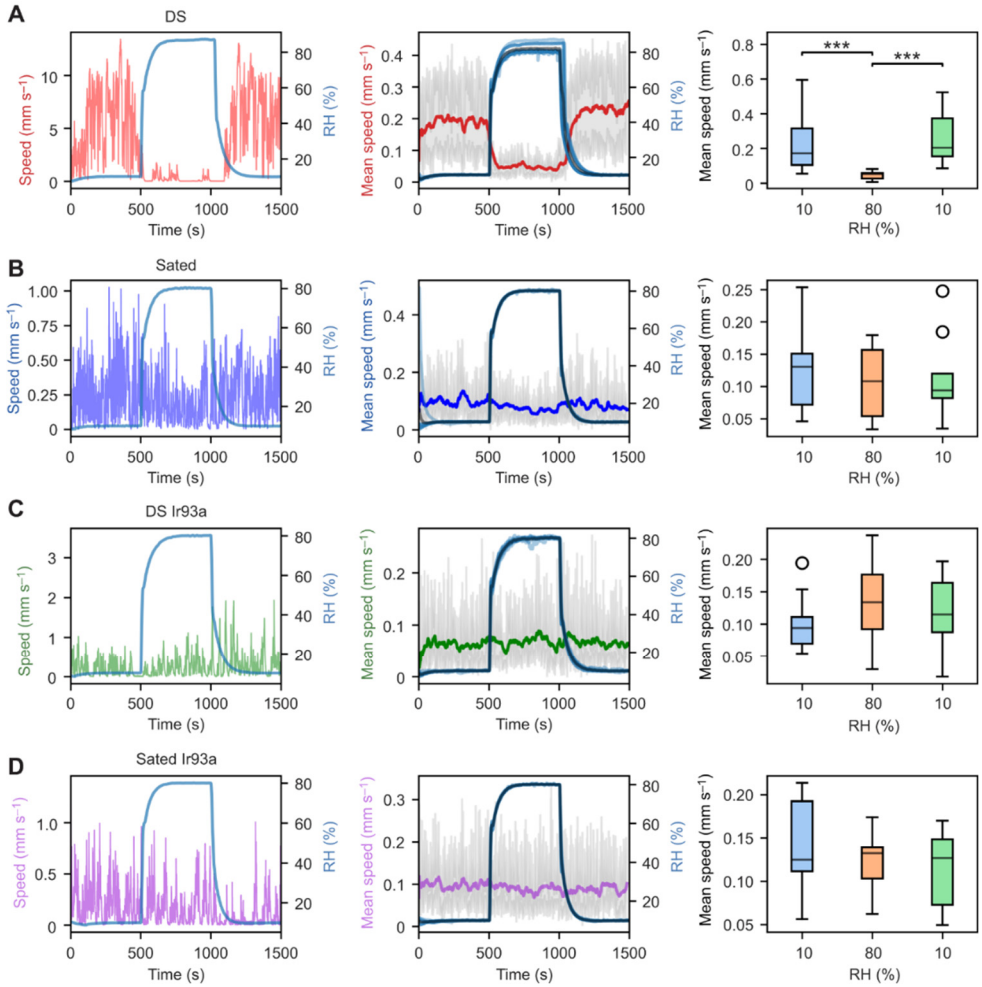

Figure 11: - Speed response to forced humidity. (A–D) Left: speed versus time graph of an individual fly from the DS (n=10; A), Sated (n=9; B), DS Ir93a (n=9; C) and Sated Ir93a (n=9; D) groups, for a step humidity function. Each humidity set point was maintained for a duration of 500 s. Centre: mean speed versus time graph calculated using all individual flies from the respective groups. Darker shades of the respective colour in the plot represent the mean normalised speed. The black curve shows the mean humidity for the given group; the grey area represents the 95% confidence interval for speed at each time point. Right: distribution of average speed of flies for each trial at each RH set point (box plots as in Fig. 3; Mann–Whitney U-test with Bonferroni correction: ***P<0.001).

These results further underscore the critical role of desiccation and starvation in shaping the flies' behavioural responses to humidity. Since the DS flies were both starved and desiccated, the observed sensitivity to humidity shifts cannot be attributed solely to either factor in isolation. To further elaborate on this, flies that

had only been starved but not desiccated (NDS) flies that were desiccated and starved but subsequently rehydrated were also tested.

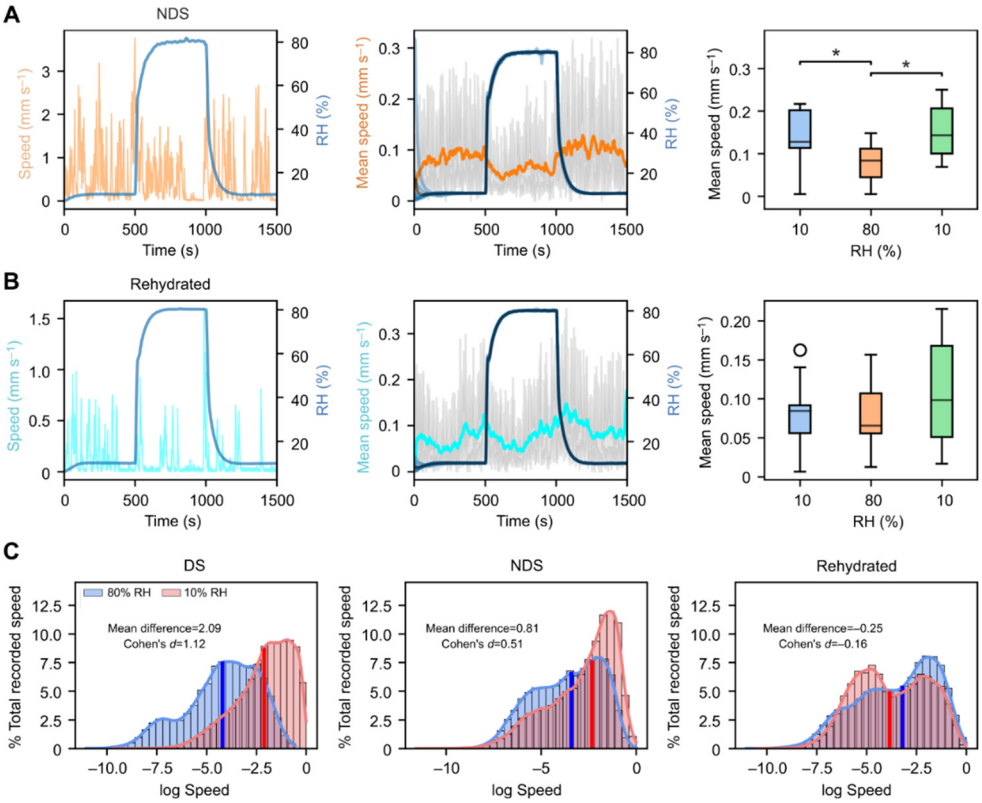

Figure 12: - Impact of different internal states on humidity-seeking behaviour. (A,B) Left: speed versus time graph of an individual fly from the starved but not desiccated (NDS; n=10; A) and Rehydrated (n=10; B) groups for a step humidity function. Centre: mean speed versus time graph calculated using all individual flies from the respective groups. Darker shades of the respective colour in the plot represent the mean normalised speed. The black curve shows the mean humidity for the given group; the grey area represents the 95% confidence interval for speed at each time point. Right: distribution of average speed of flies for each trial at each RH set point (box plots as in Fig. 3; Mann–Whitney U-test with Bonferroni correction: *P<0.05). (C) Histogram of the total recorded speed (mm s−1) at 80% and 10% RH for the DS, NDS and Rehydrated groups. The solid blue and red lines depict the mean speed for their respective RH level.

Similar to DS flies, the NDS group exhibited a significant reduction in speed at 80% RH compared to 10% RH (P<0.05). However, the Rehydrated group showed no difference in speed across humidity conditions, suggesting that rehydration neutralized the behavioural sensitivity observed in the DS flies (Figure 12 A-B). To further quantify these findings, Cohen's d values were calculated for the speed

differences between 80% and 10% RH across the DS, NDS, and Rehydrated groups, providing a measure of effect size. The DS group exhibited a large effect size (d=1.12), while the NDS group showed a moderate effect size (d=0.51). In contrast, the Rehydrated group demonstrated no significant difference, reflected by a negligible effect size (d=−0.16) (Figure 12 C).

The observed preference of 65-70% RH in desiccated and starved w^{1118} flies remains close to the values described as the preferred humidity for *D. melanogaster* in binary assays using saturated salt solutions to control humidity [74,75,93]. The similarity of reported values across methods validates the results obtained from our dynamic humidity arena and suggests that the innate humidity preference of *D. melanogaster* is incredibly robust. Such precision in identifying an optimal RH is vital for survival, shielding flies from desiccation and risks of excessive moisture, including lethal water droplets, parasite growth, and impaired respiration due to tracheal water accumulation [32–34]. The preference towards a specific humidity range disappears when the flies are either sated or lack proper humidity-sensing ability.

The observed behavioural response to humidity changes were immediate, during sudden transitions between humidity levels during the forced humidity experiments. At 10% RH, flies display increased speed, likely an escape behaviour aimed at moving from the unfavourable low-humidity environment toward the more favourable 80% RH. Both desiccation and starvation contribute to this behaviour, with desiccation playing a more dominant role as suggested by the Cohen's d values. Since starvation inherently causes some level of desiccation, the two factors interact to lower the hydration state of the fly to drive the observed humidity-dependent response.

A key mechanism underlying these state-dependent behavioural responses is the activity of HRNs, which detect RH changes and integrate them with signals about the insect's internal state. HRNs express a diverse array of neuropeptide receptors, including those for allatostatin C (AstC), diuretic hormone 31 (Dh31), short neuropeptide F (sNPF), and RYamide. These neuropeptides, which regulate physiological processes such as feeding and hydration, likely modulate HRN activity in response to the insect's hydration or starvation levels. For instance, when an insect is dehydrated or starved, elevated levels of specific neuropeptides could increase HRN activity, intensifying the drive to seek humid environments [113]. This dynamic regulation suggests that HRNs act as a bridge between internal physiological needs and behavioural responses.

The modulation of HRN activity is finely tuned by the subtype-specific expression of neuropeptide receptors, allowing for precise adjustments based on the combination of neuropeptides circulating in the insect's hemolymph. This mechanism not only enhances the flexibility of the insect's responses to humidity but also integrates with other behavioural drives, such as circadian rhythms and

reproductive behaviours [113]. For example, HRNs expressing receptors related to egg-laying or courtship may adjust sensitivity to humidity depending on the time of day or reproductive state. These mechanisms highlight the sophisticated interplay between neuropeptide signalling and HRN activity, enabling insects to prioritize survival-critical behaviours under varying environmental and physiological conditions.

The dynamic humidity arena represents a significant advancement in studying humidity-driven behaviours in insects, addressing limitations of traditional methods such as saturated salt solutions and discrete dry or moist air streams. While these conventional approaches have been instrumental in maintaining fixed humidity levels, they fail to capture the dynamic and ecologically relevant humidity variations insects encounter in their natural environments. The dynamic humidity arena overcomes this gap by delivering a precise and adjustable humidity range of 10–80% RH, enabling the creation of intricate and ecologically meaningful humidity landscapes.

One of the arena's key benefits is its ability to generate continuous humidity gradients, providing a broader and more realistic range of experimental conditions. Its real-time humidity control and tracking capabilities enhance precision and adaptability, allowing detailed investigations into insect preferences and navigation behaviours. With a low error margin of 0.2% RH, the system ensures high reliability of experimental data. Additionally, its adaptability to walking insects of varying sizes expands its applicability across species and experimental contexts [32,53,62,66,73,74,92,93,114–116]. These features make the arena a robust platform for dissecting humidity-driven behaviours and quantifying optimal humidity ranges, yielding valuable insights into insect ecology and physiology.

However, there are limitations to consider. The system requires approximately 81 seconds to reach 90% of the target humidity set point, which may restrict its use in experiments requiring rapid humidity changes or responses. Furthermore, the airflow used to deliver the humidity stimulus might inadvertently influence the insect’s sensory input by affecting antenna movement or vibration, potentially confounding behavioural interpretations. Although efforts to minimize airflow effects have been made, this remains a potential concern. Additionally, the arena is currently designed for walking insects, and significant modifications would be necessary to study humidity dependent behaviour during flight.

However, the dynamic humidity arena still offers good precision and ecological relevance, making it a valuable tool for investigating humidity-dependent behaviours. While limitations like latency and airflow effects must be accounted for, the arena's functionality provides a versatile platform for advancing the understanding of insect behaviour and response towards humidity.

Paper II: - Conserved molecular signatures of hygrosensory neurons in two dipteran species.

This study aimed to uncover the molecular basis of hygrosensation in *D. melanogaster* and *A. aegypti* through a comparative transcriptomic analysis and behavioural assay. By identifying conserved genes in the HRNs of two distinct dipteran species and investigating their roles in humidity-guided behaviour, the study provides insights into the genetic toolkit underlying this sensory system.

The ability of insects to sense humidity and temperature relies on specialized ionotropic receptors (IRs), which play key roles in hygrosensation and thermosensation. In *D. melanogaster*, the IR family members *Ir40a* and *Ir68a* are central to detecting dry and moist conditions, respectively. *Ir40a* is found in dry-responsive neurons located in chambers I and II of the sacculus, while *Ir68a* is expressed in moist-responsive neurons [73–76]. Another important player, Ir21a, is expressed specifically in hygrocool neurons in chamber I, highlighting its distinct role in sensory processing [117]. Supporting these functions, *Ir25a* and *Ir93a* are broadly expressed across all these sensory neurons, acting as co-receptors for humidity and temperature sensing [74,75].

Behavioural studies using *Drosophila* mutants reveal the specific roles of these receptors. Flies lacking *Ir40a* or *Ir68a* exhibit impaired responses to humidity, indicating their necessity for sensing dry and moist conditions. On the other hand, Ir21a mutants retain normal humidity responses but show defective thermosensation, suggesting that *Ir21a* is specialized for temperature detection. Similarly, the loss of *Ir25a* or *Ir93a* disrupts both humidity and temperature sensing, underscoring their shared roles in these sensory modalities.

This molecular framework is remarkably conserved in mosquitoes, where Ir93a is also essential for sensing both temperature and humidity. Mosquito *Ir21a* mutants show impaired temperature sensing but maintain normal humidity detection, mirroring the observations in Drosophila [118]. Additionally, *Ir40a* and *Ir68a* play analogous roles in mosquitoes, detecting dry and humid air, respectively, and are crucial for behaviours like blood-feeding and egg-laying [9,55,72,119,120].

Together, *Ir93a*, *Ir40a*, and *Ir68a* emerge as core components of the hygrosensory system, with their conserved roles across dipteran insects suggesting a fundamental evolutionary mechanism for humidity sensing. However, beyond these key receptors, the broader molecular pathways that enable the development and function of hygrosensory neurons remain largely unexplored, leaving room for further investigation into this intricate sensory system.

Transcriptomic analysis was employed to examine antennal neurons in *D. melanogaster.* This approach involves sequencing RNA to identify patterns of gene expression across neuron populations. Using clustering algorithms, antennal

neurons were grouped based on similarities in their gene expression profiles, revealing three key clusters associated with hygrosensation: clusters 14 and 27. Cluster 14 comprised hygrocool, moist, and arista temperature cells; cluster 27 contained dry cells [121–123]. These clusters expressed genes including *Ir93a*, *Ir40a* and *Ir21a*, with negligible and non-specific expression of *Ir68a*. Parallel analyses of *A. aegypti* antennal transcriptomes identified comparable neuron clusters expressing *Ir93a, Ir21a*, and *Ir40a*. As in *D. melanogaster*, *Ir68a* expression was minimal.

A comparison of the top marker genes from hygrosensory neuron clusters *in D. melanogaster* and *A. aegypti* revealed 21 conserved genes (Table 1). These included the previously described *Ir21a*, *Ir40a*, and *Ir93a*, along with 18 other genes. These novel genes spanned several functional categories, including transcriptional regulators, signalling and ion transport molecules, enzymes, and structural or adhesion-related components. Expression analysis of these genes in *D. melanogaster* sacculus neurons showed distinct patterns across subtypes, indicating their specialized roles in hygrosensation.

Table 1: Conserved Genes in Hygrosensory Neurons of *D. melanogaster*

Gene	Category	Function
Seven-up (svp)	Transcription factor	Nuclear receptor
Ribbon (rib)	Transcription factor	BTB/POZ domain nuclear factor
LIM homeobox 1 (Lim1)	Transcription Factor	LIM homeodomain transcription factor.
nubbin (nub)	Transcription Factor	POU/homeodomain transcription factor.
spalt-related (salr)	Transcription Factor	Zinc finger transcriptional repressor.
homothorax (hth)	Transcription Factor	Homeodomain transcription factor.
disco-related (disco-r)	Transcription Factor	C2H2 zinc-finger transcription factor.
CG42594	Ion channel	Potassium channel
CG32683	Cellular Signaling	Arrestin
Ir21a	Receptor	Essential for thermosensation
Ir40a	Receptor	Involved in dry air detection
Ir93a	Receptor	Essential for functional thermos and hygrosensation
5-HT7	Receptor	Serotonin receptor
olf413	Enzyme	Dopamine beta-monooxygenase involved in catecholamine synthesis.
CG9743	Enzyme	Enzyme with stearoyl-CoA desaturase activity.
CG3655	Enzyme	Glycosyltransferase enzyme.
Kif19A	Structural/Adhesion Component	Microtubule-associated motor protein.
CG14274/witty	Structural/Adhesion Component	GPI-anchored protein.
CG32432	Structural/Adhesion Component	GPI-anchored protein.
Dscam4	Structural/Adhesion Component	Cell adhesion molecule.
fred	Structural/Adhesion Component	Cell adhesion molecule.

To investigate their functional roles in behaviour, a subset of these genes was further tested for responses to humidity changes in the dynamic humidity arena. Among these were the serotonin receptor *5-HT7*, kinesin motor protein *Kif19A*, and the transcription factor *nub* along with *Ir21a*.

Behavioural assays in *D. melanogaster* mutants further confirmed the functional significance of three conserved genes. Flies with mutations in *Ir21*a displayed normal humidity-guided behaviour, suggesting that the function of hygrocool cells in sacculus chamber I may not be critical under the tested conditions or that their role could be compensated by alternative pathways. In contrast, flies with mutations in *5-HT7* or *Kif19A* showed no behavioural response to humidity changes, demonstrating that these genes are essential for normal hygrosensory behaviour. Additionally, flies with a hypomorphic mutation in *nub* (*nub*^{2}) exhibited

significantly impaired humidity-guided behaviour, indicating a key role for *nub* in this sensory system (Figure 14).

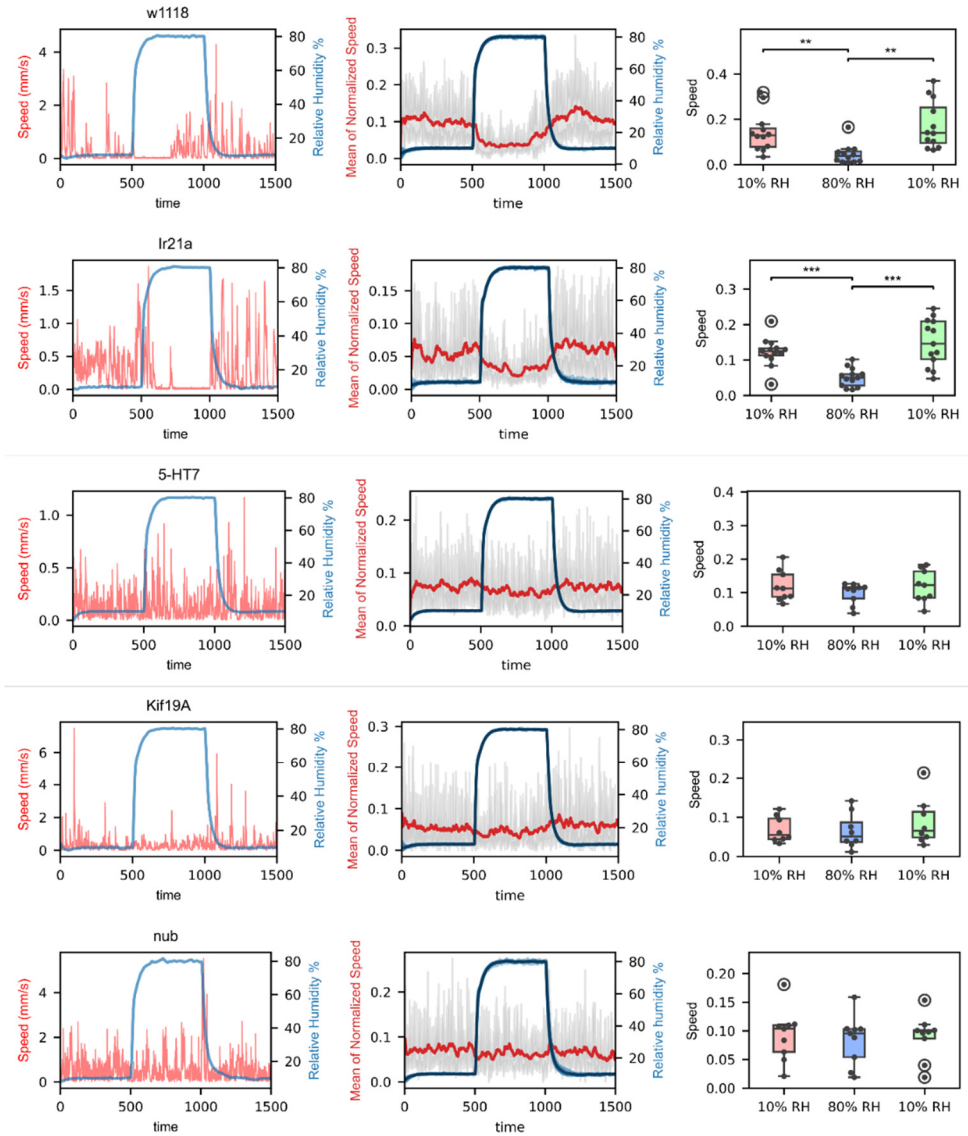

Figure 13: - Genetic dissection of humidity-sensing behaviour. (A-E) Behavioural responses to humidity changes in control and mutant flies. Red lines show mean of normalized speed; gray shading indicates 95% confidence interval. Blue lines represent RH changes (10% → 80% → 10% RH). n = 8-13 flies per genotype. Bootstrapping was used to assess statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Box plots show median (line), interquartile range (box), upper and lower quartile (whiskers), individual fly responses (dots) and outliers (circled dots).

Overall, the study identified a conserved molecular toolkit for hygrosensation in dipteran insects, encompassing 21 genes with diverse functional roles. These genes span transcriptional regulation, structural organization, and sensory signaling, highlighting the coordinated action of diverse molecular pathways in enabling insects to detect environmental humidity. Their conservation across dipteran species, separated by over 200 million years of evolution, underscores their fundamental role in hygrosensation. Behavioural assays highlighted the importance of *5-HT7*, *Kif19A*, and *nubbin* for proper humidity sensing in *D. melanogaster*. These findings provide a foundation for further exploration of the molecular mechanisms underlying hygrosensation across insect species.

A set of seven conserved transcription factors (*svp, rib, Lim1, nub, salr, hth,* and *disco-r*) plays a crucial role in establishing and maintaining the identity of hygrosensory neurons. These factors are involved in both developmental processes and adult neuron function. For instance, *hth* is essential for antennal development and segmental identity, while *Lim1* contributes to appendage specification [124,125]. Beyond development, these transcription factors continue to regulate neuron subtype identities, with *salr* marking moist-sensitive cells and *nub* associated with hygrocool and temperature-sensitive neurons. This combinatorial expression likely forms a regulatory code defining the molecular and functional properties of each sensory neuron subtype. The conservation of this transcriptional blueprint between flies and mosquitoes suggests a shared strategy for maintaining sensory neuron function across species.

The cell adhesion molecules *fred* and *Dscam4* contribute to the precise structural organization of hygrosensory neurons. *Fred*, which is specifically expressed in moist-sensitive cells, likely helps maintain their architecture within the sacculus [126,127]. On the other hand, *Dscam4* is found in one subtype of dry-sensitive cells and may work alongside its paralog *Dscam2* to organize distinct subtypes of dry cells in different sacculus chambers [128]. These adhesion molecules mediate neuronal targeting and synaptic connectivity, ensuring the proper circuit architecture required for sensory processing [129].

Humidity detection relies heavily on ionotropic receptors (IRs), and two conserved GPI-anchored proteins, *CG14274/witty* and *CG32432,* play critical roles in organizing and regulating these receptor complexes. Similar to their roles in other contexts, *CG14274* facilitates receptor aggregation, while *CG32432* modulates receptor stability and gating properties [130–132]. These functions are vital for ensuring efficient sensory transduction in hygrosensory neurons.

Mutations in *Ir21a*, despite its known role in hygrocool cells, did not affect the flies' humidity responses in the behavioural assays. This result aligns with previous studies, suggesting that the hygrocool cells in sacculus chamber I may not be essential for guiding humidity-dependent behaviour under these assay conditions [74,75]. Alternatively, other neural pathways or mechanisms may compensate for

the absence of *Ir21a* function, maintaining normal humidity responses. These findings underscore the redundancy and plasticity inherent in sensory systems.

In contrast, the serotonin receptor gene *5-HT7* was found to be critical for normal humidity-guided behaviour, as *D. melanogaster* with mutations in this gene exhibited significantly impaired responses. Previous research has established *5-HT7's* role in coordinating sensory input with physiological processes, such as translating gustatory stimuli into digestive responses [133]. In the context of hygrosensation, serotonin signalling through *5-HT7* may modulate the excitability of hygrosensory neurons, fine-tuning their sensitivity to humidity changes. Given serotonin's role as a diuretic hormone in insects [134], *5-HT7* may play a role in humidity sensing by potentially modulating the excitability of hygrosensory neurons to fine-tune their responses to environmental moisture changes, aiding the fly in balancing water loss and desiccation risk in response to environmental humidity and feeding state.

The kinesin motor protein *Kif19A* emerged as a critical factor for humidity sensing in *D. melanogaster*. Mutant flies lacking functional *Kif19A* exhibited significant impairments in behavioural responses to humidity changes, underscoring its essential role in sensory neuron function. As a kinesin-8 family motor protein, *Kif19A* regulates ciliary length through microtubule depolymerization, a function essential for maintaining proper ciliary architecture and sensory function [135]. Its activity likely ensures that cilia in dry cells remain structurally optimized for humidity detection. Additionally, *Kif19A* may facilitate the transport of sensory components, such as ionotropic receptors, to ciliary tips, enabling efficient sensory transduction. Therefore, by preserving ciliary integrity and facilitating the localization of key sensory components, *Kif19A* ensures the proper function of sensory neurons essential for humidity detection.

The transcription factor *nubbin* was also shown to be necessary for humidity-guided behaviour, as flies with the hypomorphic nub^{2} mutation displayed significant impairments in humidity sensing behaviour. *Nubbin* likely plays a multifaceted role in the development and maintenance of sensory neurons. *Nubbin's* known redundancy with its paralog *pdm2* in wing development, regulated by a shared enhancer element, suggests a parallel role in sensory neurons [136]. This enhancer is also active in HRNs in the adult antenna [137], indicating that *nubbin* may similarly regulate key aspects of sensory neuron function, such as maintaining receptor expression and coordinating ion channel activity essential for functional humidity sensing.

This study highlights the critical interplay between molecular components and behaviour in the context of hygrosensation in *D. melanogaster* and *A. aegypti*. By combining transcriptomic analyses and behavioural assays, we identified a suite of conserved genes essential for humidity sensing, underscoring their specialized roles in this sensory system. The behavioural results emphasize the importance of genes

such as *5-HT7*, *Kif19A*, and *nubbin*, whose disruption significantly impaired humidity-guided responses, highlighting their fundamental role in ensuring proper sensory neuron function and behavioural adaptation to environmental humidity. These findings not only deepen our understanding of the molecular basis of hygrosensation but also pave the way for further exploration of how conserved genetic pathways shape sensory behaviours across insect species.

Paper III: - Humidity -dependent structural adaptations of *Drosophila melanogaster* hygrosensilla

This study investigated the structural differences of hygrosensilla in *Drosophila melanogaster* under two different humidity conditions. By combining Serial block face scanning electron microscopy and U-Net based segmentation model, the study revealed chamber-specific structural differences in the hygrosensilla between samples exposed to high and low humidity conditions. A summary of the findings is presented below.

To systematically analyse the structural properties of individual sensilla, a deep learning-based U-Net segmentation model was implemented which achieved an accuracy of 95% and an Intersection over Union (IoU) score of 0.854 on the validation data set (Figure 14 A). The model demonstrated robust performance despite the sacculus's complex structure and the smaller region of interest when compared to the background and facilitated a reliable segmentation with all the tested images (Figure 14 B).

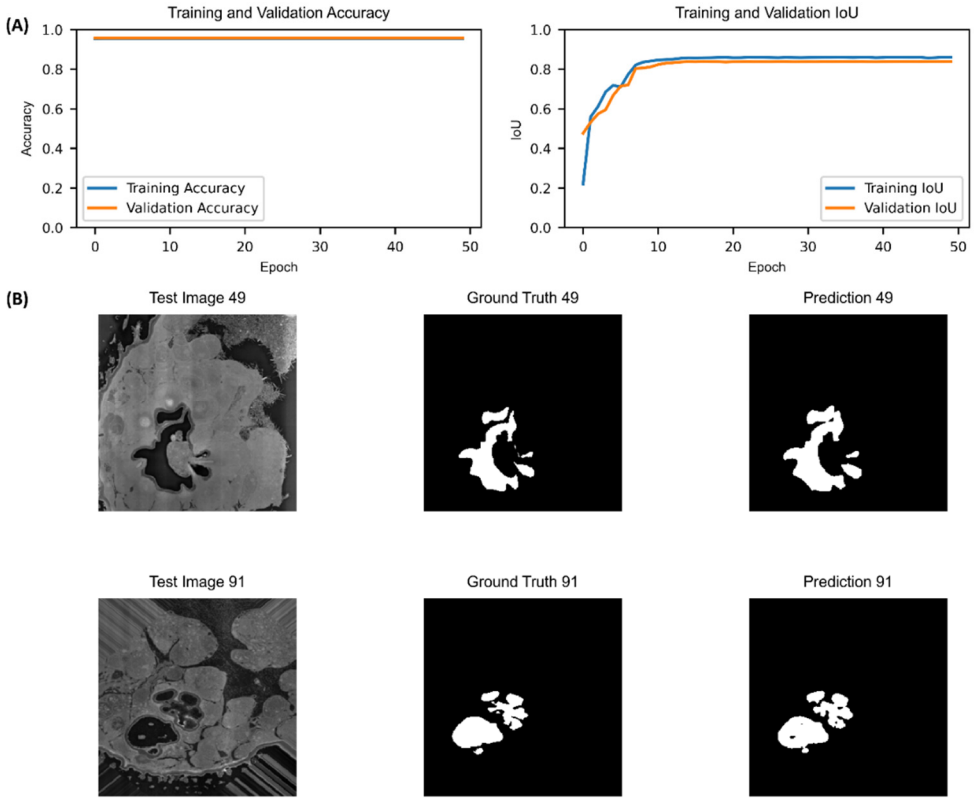

Figure 14: - Evaluation and application of the U-Net model for sacculus segmentation. (A) Model performance metrics, showing Accuracy (Left) and Intersection over Union (IoU) (Right), for both training and validation datasets. (B) Segmentation results using the trained U-Net model on test data, displaying the test input (Left), the corresponding manually labelled ground truth (Center), and the model's prediction (Right).

Using the segmented contours, the 3D structure of the sacculus was reconstructed (Figure 15), revealing four distinct types of sensilla distributed across the sacculus's three chambers. In Chamber 1, the sensilla were elongated and slender, tapering consistently toward the tip, while those in Chamber 2 were shorter and wider, with a broad base and less pronounced tapering. Chamber 3 contained two types of elongated sensilla, with the lower section featuring notably wider sensilla compared to the upper section (Figure 16 A-B). These structural characteristics were consistent across both the humidity conditions but exhibited noticeable differences in dimensions between high and low RH samples.

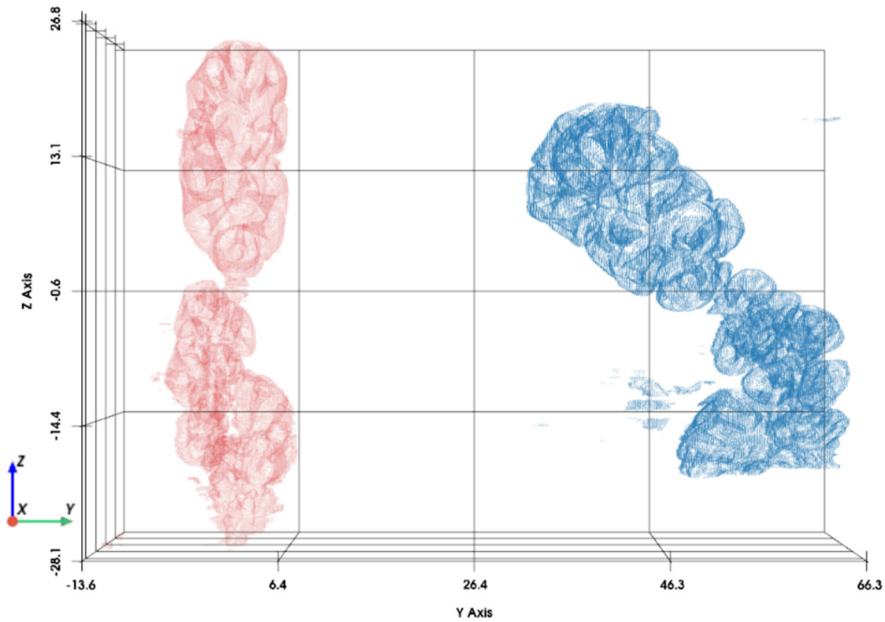

Figure 15: - 3D structure of the sacculus. Contours extracted from segmented images were used to generate point clouds, visualizing the 3D structure of the sacculus. The red point cloud represents the sacculus under low humidity conditions, while the blue point cloud corresponds to the sacculus under high humidity conditions.

In the high humidity dataset (80% RH), sensilla in Chamber 1 had an average width of 3.36 µm, 0.77 µm wider than those in the low humidity dataset (26% RH), where the average width was 2.59 µm. Similar patterns were observed in Chambers 2 and 3, where sensilla under high humidity conditions were consistently wider, with statistically significant differences in median full width at half maximum (FWHM) across all chambers. The largest difference was in Chamber 1, showing a 0.4 µm increase ($p < 0.05$), while Chambers 2 and 3 exhibited increases of 0.33 µm each ($p < 0.01$ and $p < 0.05$, respectively) (Figure 16 C, top and center).

A mixed-effects model analyzing the relationship between sensilla width and height revealed distinct humidity-dependent tapering patterns (Figure 17 C, bottom). In Chamber 1, sensilla under high humidity showed a sharper tapering, with a slope of -0.472 compared to -0.403 under low humidity, indicating a structural adaptation that may enhance sensitivity of the hygrosensory neurons. In contrast, Chambers 2 and 3 exhibited steeper tapering under low humidity conditions, with slopes of -1.120 and -0.652, respectively, compared to -0.959 and -0.541 in high humidity. These findings highlight the dynamic structural plasticity of sensilla in response to environmental humidity, suggesting optimized sensory function through differential tapering across chambers.

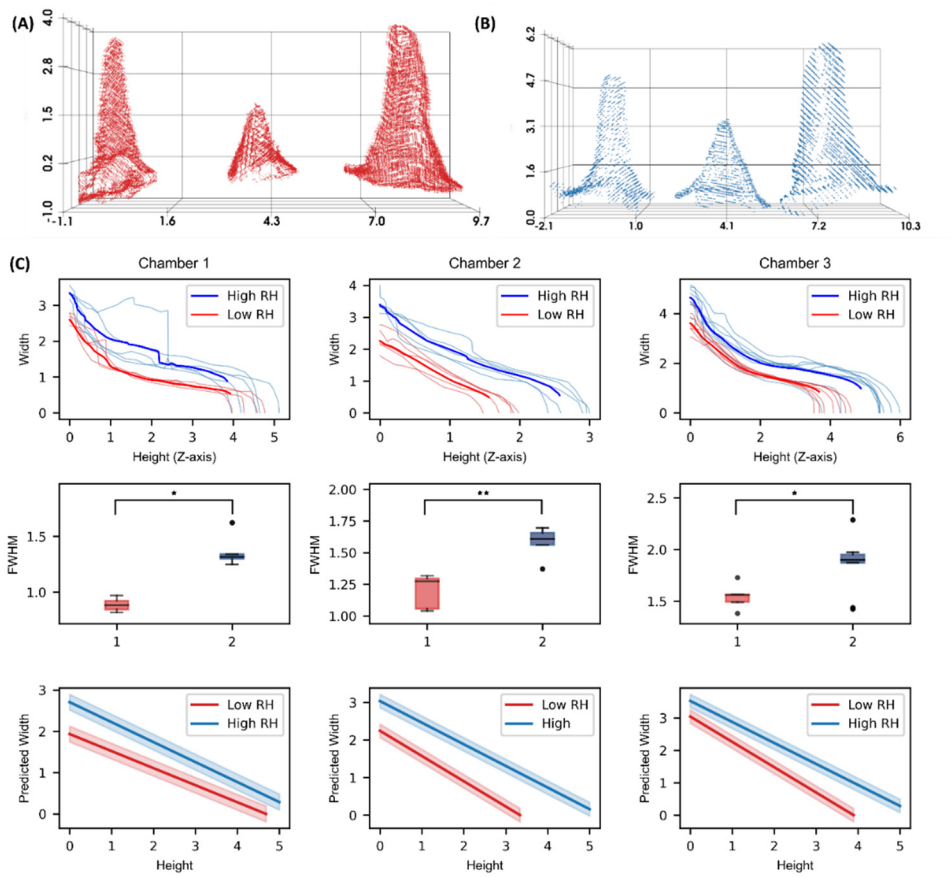

Figure 16: Structural comparison of D. melanogaster sensilla across humidity conditions. (A) Sensilla point clouds from low humidity (26% RH) and (B) high humidity (80% RH) samples, oriented and aligned for Chambers 1, 2, and 3 from left to right. (C) Top: Length vs. width profiles for each identified sensillum in Chamber 1 (Left), Chamber 2 (Center), and Chamber 3 (Right), with low humidity samples in red and high humidity samples in blue; darker curves represent the mean length vs. width profiles for each condition. Middle: Full width at half maximum (FWHM) values for each identified sensillum, calculated by determining the height of each sensillum and locating the corresponding width at half this height; statistically significant differences (Mann Whitney U test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) are observed between high and low RH conditions across all chambers. Bottom: Mixed-effects model of the length vs. width profiles. Solid lines represent calculated profiles, with shaded areas indicating the 95% confidence intervals. Notably, in Chamber 1, high humidity sensilla display a steeper taper (slope: -0.472) compared to low humidity (slope: -0.403). In contrast, sensilla in Chambers 2 and 3 exhibit a steeper taper under low humidity (slopes: -1.120 and -0.652, respectively) than under high humidity (slopes: -0.959 and -0.541).

Although the results reveal significant differences in the width of sensilla between the dry and humid samples, it remains challenging to attribute these differences solely to the humidity conditions during sample preparation. The variations might also stem from individual differences in sensilla size between the two selected flies. To draw definitive conclusions, it is essential to analyze additional samples and minimize the influence of individual variability.

If the observed differences are indeed caused by humidity changes, the findings would support the mechanical hygrometer model as a key mechanism for humidity sensation. Unlike olfactory sensilla, which detect airborne molecules through cuticular pores, hygrosensilla lack pores and are likely specialized for detecting physical stimuli associated with humidity changes [9].

The observed increase in sensilla width under high humidity conditions suggests that structural changes in the sensilla play a pivotal role in humidity sensation. These changes likely activate mechanosensors in the plasma membrane of HRNs, which extend their sensory cilia into the hygrosensillum and maintain close contact with the cuticular wall [72]. The differential expression of cuticle-associated proteins, such as *vermiform* in dry-sensing neurons and *fred* in moist-sensing neurons, highlights a potential structural link between HRNs and the sensilla [127,138,139]. These proteins, integral to chitin metabolism and interaction, may contribute to the specialized cuticular architecture essential for hygrosensory function. Additionally, the complementary expression of nicotinic acetylcholine receptor subunits α6 and α7 in dry- and moist-sensing HRNs, previously implicated in mechanotransduction, supports the involvement of a mechanosensory component in humidity sensing [140,141].

However, the mechanosensory model must reconcile with electrophysiological data showing that the responses of dry and moist cells to humidity fluctuations increase with rising temperature [142]. This apparent contradiction can be explained by the role of ephaptic coupling, a non-synaptic mechanism by which sensory neurons housed within the same sensillum electrically inhibit each other [143–145]. Ephaptic inhibition, or ephaptic coupling, is a form of non-synaptic lateral inhibition that occurs between neurons in close proximity. Unlike traditional synaptic communication, ephaptic inhibition relies on extracellular electrical field changes to modulate neuronal activity. This mechanism has been observed in *Drosophila* olfactory receptor neurons (ORNs) housed within the same sensory hair, or sensillum, where the activation of one ORN suppresses the activity of neighbouring ORNs, fine-tuning sensory responses. The process begins with ionic fluxes generated by neuronal activity, which alter the local extracellular potential. These changes influence the excitability of neighbouring neurons via electrical field effects. Specifically, when one ORN is activated, it reduces the transepithelial potential, a driving force for odor-induced transduction currents. This reduction shunts ionic currents away from adjacent ORNs, inhibiting their activity. The strength of this inhibition depends on the magnitude of the local field potential

(LFP) generated by an ORN, with stronger LFP responses leading to greater suppression of neighbouring neurons. For ephaptic inhibition to function effectively, specific conditions are required, including high extracellular resistance, high neural membrane density, close neuronal proximity, and a lack of insulating structures such as myelin. These conditions amplify electrical field effects, making ephaptic coupling especially prominent in environments where neurons are tightly packed, such as in *Drosophila* sensilla.

In the case of hygrosensation, the hygrocool neuron, consistently found in close proximity alongside dry and moist cells, within an insulated sensillum likely modulates their responses through temperature-dependent ephaptic inhibition. As temperature decreases, increased activity in the hygrocool cell may suppress the responses of the dry and moist cell, thereby aligning humidity sensation with environmental conditions [146]. While speculative, this model offers a functional explanation for the conserved grouping of these three cell types in hygrosensilla, emphasizing the integration of mechanosensory and temperature-dependent regulation in humidity sensation.

Conclusion and future perspective

This thesis investigated hygrosensation in *Drosophila melanogaster* through a multidisciplinary approach including behavioural, molecular and structural methods. It includes the development of novel methods for both behavioural and structural analysis of hygrosensation. The integration of these three complementary approaches provided unique insights into how insects detect and respond to humidity. *D. melanogaster* is as a powerful model system for such research, offering sophisticated genetic tools and relatively simple neural circuits that can reveal fundamental principles of sensory processing. These principles can inform our understanding of human wetness sensation, which, like in insects, involves the integration of multiple sensory inputs. Additionally, insights gained from studying humidity sensation in *D. melanogaster* can shed light on how disease vectors such as mosquitoes use humidity cues to locate human hosts, potentially leading to improved strategies for preventing disease transmission.

Paper I demonstrated that desiccated and starved flies exhibit a strong preference for 65–70% RH and show rapid behavioural responses to sudden humidity changes. These preferences were absent in sated flies and flies lacking functional humidity sensors, highlighting the critical role of the internal state and hygrosensory system in guiding behaviour.

The dynamic humidity arena developed in this study represents a significant advancement in methodology. Traditional studies have relied on binary choice assays using either saturated salt solutions or simple 'dry' and 'moist' air streams, which offered limited control over humidity levels [74,75,92,93]. In contrast, the dynamic humidity arena enables precise control of humidity across a continuous range (10-80% RH), better reflecting the environmental conditions insects encounter in nature. This technical advance bridges an important gap between behavioural studies and electrophysiological findings, which have shown that HRNs can detect humidity changes as small as 1% RH [66,67]. However, our study could not confirm a behavioural relevance of such a fine-grained sensory ability.

Understanding how internal state influences humidity preferences has important implications for pest control and disease vector management. In so-called anautogenous female mosquitoes, which require blood meals for egg development, behavioural responses to sensory cues including humidity are tightly regulated by their physiological state [147]. Newly emerged females initially show limited host-seeking behaviour, but develop full responsiveness to host cues, including humidity, over 4-10 days post-emergence [55]. After blood-feeding, females enter a refractory

period where they stop responding to host cues through a two-phase inhibition process, and instead become more responsive to cues that signal suitable oviposition sites. This state-dependent switching between host-seeking and egg-laying behaviours demonstrates how internal state can fundamentally alter an insect's response to sensory cues, such as humidity, that could be exploited for vector control strategies. Adapting the dynamic humidity arena to use with mosquitoes would provide a framework for studying such processes in these disease vectors.

The functionality of the dynamic humidity arena could be significantly enhanced by integrating temperature modulation and optogenetic tools. These additions would enable precise manipulation of specific HRNs to explore their roles in humidity-dependent behaviours. For instance, optogenetics could selectively activate or inhibit specific HRNs, providing insights into the contribution of individual neurons or circuits to humidity sensation [148,149]. Temperature control would further allow the investigation of how humidity-guided behaviours are modulated at different temperatures. This could provide insights of what variable it is that insects detect, the temperature dependent variable Relative humidity or some other variable of humidity. Together, these approaches could also facilitate the study of the hypothesized ephaptic coupling mechanism within the sensilla. By dynamically varying temperature and humidity while manipulating neuronal activity, it would be possible to assess how electrical interactions between adjacent neurons influence their response properties.

Paper II revealed a conserved molecular toolkit for hygrosensation across dipteran species, identifying 21 genes shared between *D. melanogaster* and *A. aegypti*. The discovery of these conserved elements not only provides insights into the evolution of hygrosensation but also offers potential targets for vector control strategies. The identified genes span multiple functional categories, including transcriptional regulators, signaling molecules, and structural components

The essential role of *Kif19A* and cell adhesion molecules such as *Dscam4* and *fred* points to the importance of proper sensory cilium architecture and dendrite-cuticle interactions in hygrosensation. As a microtubule-depolymerizing kinesin, *Kif19A* could maintain optimal ciliary length and organization of intracellular signaling molecules, crucial for sensory transduction. In mice, kinesins have such function in nociceptors, modulating the duration of pain signaling [150]. The identification of multiple cell adhesion molecules suggests mechanical coupling between sensory dendrites and the hygrosensilla cuticle, potentially supporting the mechanical hygrometer model of humidity detection. This mechanical aspect of humidity sensation shows interesting parallels with human wetness perception, where mechanosensitive pathways play a crucial role in detecting moisture.

The identification of *5-HT7* as essential for a behavioural response to humidity provides a link between sensory processing and physiological state. As a serotonin

receptor expressed in HRNs, *5-HT7* may serve as a key modulator that adjusts humidity sensitivity based on the insect's hydration status [151]. This aligns with serotonin's known role as a diuretic hormone in insects, suggesting a direct pathway through which internal state could influence sensory processing. Future studies combining calcium imaging with manipulation of both hydration state and serotonergic signalling could reveal how *5-HT7* modulates hygrosensory neuron activity. Such experiments would help uncover the neural mechanisms that allow insects to adjust their humidity preferences based on physiological needs, potentially revealing fundamental principles of state-dependent sensory modulation.

Several transcription factors identified in the screen, including *nubbin*, likely play crucial roles in establishing and maintaining HRN identity. Future studies could explore how these factors regulate the expression of downstream effector genes and maintain the specialized functions of hygrosensory neurons throughout the insect's life.

From an applied perspective, these findings open new avenues for vector control. The conservation of these genes in *A. aegypti* suggests they could be targeted to disrupt mosquito host-seeking behavior, which relies heavily on humidity cues. Manipulating serotonin signaling has already been discussed as a vector control strategy in mosquitoes [152].

Looking forward, a key priority would be to elucidate the precise molecular mechanisms by which these genes contribute to humidity sensation. Figuring out the transduction mechanism of HRNs remains one of the bigger unsolved questions in the field. Such studies would not only advance our understanding of sensory biology but could also reveal new targets for controlling disease vectors while potentially offering insights into human moisture sensation.

Paper III examined structural differences in hygrosensilla between high and low humidity conditions, showing chamber-specific variations in both width and tapering patterns. While these findings suggest support for the mechanical hygrometer model, the limited sample size requires careful interpretation. The observed expansion of sensilla width under high humidity conditions, particularly in Chamber I, indicates potential structural adaptations that could enable mechanosensory transduction of humidity changes. This connects with the molecular findings from Paper II, where *Kif19A* and cell adhesion molecules were found to be essential for proper hygrosensory function, suggesting precise structural requirements for humidity sensation.

The technical challenges of sample preparation remain significant. The plunge-freezing process and fixation procedure must preserve structural details under specific humidity conditions, requiring precise timing and careful handling. Future studies need to examine multiple specimens across different humidity levels and

time points to determine whether the observed differences reflect humidity-dependent changes rather than individual variation. The deep learning segmentation pipeline developed for this study provides an efficient method for analyzing sensory structures and could facilitate the examination of larger sample sizes needed to validate the current findings.

Future work combining structural analysis with targeted manipulation of mechanosensory components could help validate the mechanical hygrometer model. Examining how mutations in *Kif19A*, *fred* or *Dscam4* affect hygrosensilla structure could reveal their roles in maintaining sensory architecture. Additionally, developing methods for real-time observation of structural changes in response to humidity shifts would provide insights into hygrosensation mechanisms, though this requires substantial technical advances in sample preparation and imaging methods.

The evidence presented, while intriguing, represents an initial step toward understanding the structural basis of humidity sensation. Additional studies using complementary approaches and larger sample sizes will be necessary to establish a definitive link between structural changes and hygrosensory function.

Together, these studies offer a comprehensive understanding of hygrosensation, linking behavioural preferences, conserved molecular pathways, and potential structural adaptations in response to humidity. By integrating findings across multiple levels - from molecular mechanisms to structural adaptations and behavioural outputs - this thesis provides a solid foundation for future research into sensory mechanisms and highlights the evolutionary and functional complexity of hygrosensation in insects. The methodologies and findings presented here open new avenues for investigating similar sensory systems in other species and may contribute to our broader understanding of how organisms detect and respond to environmental cues. As climate change continues to alter global humidity patterns, understanding how insects detect and respond to humidity becomes increasingly important for both basic research and applied fields such as agriculture and public health. This thesis lays the groundwork for such future investigations while providing immediate insights into the fascinating complexity of insect sensory systems.

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