

Exploring Perceptions and Reasoning in Scientific and Non-Scientific Communities on the Ethical Treatment of Animals in Octopus Research

Focusing on the paralarval *Octopus vulgaris*



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Summary

This thesis explores how biologists and non-scientists evaluate the ethical status of animal research, with particular attention to how their perceptions and reasoning are shaped by scientific information. The focus lies on ethical judgments concerning the use of *Octopus vulgaris* paralarvae, which fall under the protection of European Union Directive 2010/63/EU, in scientific research. This directive applies to all “live cephalopods” used in research likely to cause pain, suffering, or distress. While legally included, paralarvae remain ethically and scientifically ambiguous due to limited data on their capacity for pain. Should paralarvae be protected on the basis of potential sentience, even in the absence of conclusive data? Or should ethical treatment be reserved only for organisms whose capacity for pain has been clearly demonstrated? This thesis investigates how individuals interpret such distinctions and how scientific information influences their ethical judgments.

The primary component of the research is an experimental philosophy study using an online survey. The study targeted three distinct participant groups: cephalopod biologists, biologists working with other organisms, and non-biologists (laypeople). Participation was entirely voluntary. All participants were first asked to complete a set of demographic questions and were then presented with one of three versions of a short research abstract describing a gene expression study examining nociception-related genes in *O. vulgaris* paralarvae. The content of the abstract was inspired by a real gene expression study also conducted as part of this thesis. Using hybridization chain reaction (HCR), the study examined the expression of six genes—*Kcnk1*, *Trpa1*, *Ano1/2*, *Asic1*, *Oprm2*, and *Piezo2*—associated with nociception and mechanosensory signaling. These genes were investigated in *O. vulgaris* paralarvae at 5 and 40 days post-hatching. The findings revealed that several nociception-related genes were expressed in region-specific and developmentally dynamic patterns. For instance, *Asic1* showed increasing expression in central neural structures, *Piezo2* displayed patterns consistent with mechanosensory function, and *Oprm2* extended known opioid signaling regions. These results suggest that paralarvae possess a molecular framework for nociception. However, as nociception does not imply conscious experience, this evidence does not decisively establish the capacity for pain. For the survey, this biological information was adapted into three abstract versions that each described a different experimental outcome, aiming to assess how scientific information influenced ethical reasoning. The first version reported the expression of nociception-related genes, the second stated that no such expression was found, and the third conveyed scientific uncertainty. Participants were then asked to answer a broad set of questions exploring their ethical perceptions and reasoning, including the ethical acceptability and societal relevance of the research, their belief in whether the animals were capable of experiencing pain, and their confidence in that pain assessment. These questions were asked first with respect to *O. vulgaris* paralarvae and subsequently regarding adults.

The survey results showed that participants' ethical judgments were not significantly influenced by the vignettes. Whether participants read that nociception-related genes were expressed, absent, or uncertain, their responses were statistically similar. This suggests that the information was either not seen as morally decisive or was difficult to interpret, highlighting challenges in communicating complex biological findings to diverse audiences. The strongest predictor of ethical judgment was belief in paralarval pain. Participants who believed that paralarvae could feel pain were more likely to view the research as unethical and less inclined to justify it based on societal benefit. This belief was most common in the group exposed to the uncertainty scenario, suggesting that ambiguity may prompt a more cautious moral stance aligned with the precautionary principle. Ethical judgments also varied by participant background. Cephalopod biologists were least likely to find the research unethical, likely due to familiarity with the species and trust in ethical procedures, while biologists working with other organisms showed intermediate levels of concern. Laypeople were most ethically cautious, challenging assumptions that the public undervalues invertebrate welfare. However, it is also worth noting that the responses concerning *O. vulgaris* paralarvae may not be representative of public attitudes toward all invertebrates. Gender differences were also observed. Male participants more frequently prioritized societal relevance, whereas female participants tended to express greater caution and were more likely to support further investigation into the potential for paralarval pain. This pattern may reflect broader gender-based differences in moral decision-making, as women are generally more empathic, more risk-averse, and less accepting of ethically ambiguous practices. Familiarity with research ethics did not significantly influence participants' evaluations, suggesting that formal knowledge of ethics may not always translate into applied moral reasoning. However, as this familiarity was self-rated rather than formally assessed, participants may have overestimated their knowledge, introducing potential bias into the results. Finally, confidence in pain assessment was partly linked to academic experience. Participants with high academic experience expressed the most confidence, as expected. Yet, non-biologists reported similarly high confidence, exceeding that of participants with moderate experience, possibly due to unawareness of the biological complexity and an overestimation of their understanding, consistent with the Dunning–Kruger effect.

In conclusion, this thesis provides both empirical and ethical insights. It contributes novel developmental data on nociception-related gene expression in *O. vulgaris* paralarvae, suggesting that the molecular basis for sensory detection is already present in early life stages. Additionally, it shows that ethical reasoning is influenced more by prior beliefs, disciplinary background, and gender than by scientific content or familiarity with research ethics. Scientific uncertainty may encourage ethical caution, but scientific information, if poorly understood, may not meaningfully shape moral evaluations. These findings highlight the need for more accessible science communication and ethics education in animal research, particularly when policy decisions intersect with uncertain biological evidence.

1. Introduction

Animal models have long played a role in scientific research, with their use becoming increasingly prevalent since the 19th century (Fernandes & Pedroso, 2017; Mukherjee et al., 2022). Today, non-human animals are commonly employed as experimental subjects in most medical research institutions worldwide (Kiani et al., 2022; LaFollette, 2025). This approach has proven valuable for advancing our understanding of human diseases and investigating possible therapeutic interventions (Franco, 2013; Kiani et al., 2022). However, animal research is not without constraints. It is subject to important limitations, many of which are shaped by ethical considerations, including legal frameworks, societal attitudes, and efforts to identify humane and scientifically valid research alternatives (Mukherjee et al., 2022). Government regulations play a key role in limiting the potential for injury, pain, or distress in animal research, imposing strict guidelines on both researchers and institutions (Vandewoude & Rollin, 2009; Mukherjee et al., 2022). At the same time, legislation in many modern nations mandates that certain tests, such as those for vaccines or lethal doses, must first be conducted on animals before proceeding to human trials (Kooijman, 2013; Mukherjee et al., 2022). Beyond legal frameworks, societal acceptance and relevance significantly shape the scope of animal experimentation, particularly because it often involves the use of public funds (Vandewoude & Rollin, 2009; Mukherjee et al., 2022). Ethical perceptions also vary among the public; for example, some individuals attribute greater moral weight to the welfare of dogs compared to other animals commonly used in research, such as pigs, rats, fish, or mice (Mukherjee et al., 2022). A central issue in the ongoing debate about animal experimentation is the ethical justification for using animals in scientific research (Paixão & Schramm, 1999; Kiani et al., 2022). In response to ethical concerns, some non-human animals have been granted legal protections that reflect a form of moral rights (Directive 2010/63/EU, 2010; Mukherjee et al., 2022; Gruen & Monsó, 2024). Since protections are limited to specific species, several important questions arise: Why does ethical legislation extend only to certain non-human animals? On what basis do we assign moral consideration and rights? And is it ethically justified to draw the line where we currently do?

A being is morally considerable if it can be wronged (Gruen & Monsó, 2024). Numerous criteria have historically been proposed to define who qualifies for moral consideration, including speciesism, human exceptionalism, personhood, agency, and sentience (Gruen & Monsó, 2024). In this study, particular attention will be given to sentience—the capacity of an animal to subjectively experience and consciously perceive a range of affective states, including pleasure, pain, satisfaction, and suffering (Broom, 2007; Gruen & Monsó, 2024). Over the past few decades, a growing body of research has significantly deepened our understanding and awareness of animal sentience (Duncan, 2006; Kiani et al., 2022). Although much of the early research on sentience focused on mammals and birds, emerging evidence now supports sentience-based arguments for recognizing the moral status of cephalopods (Godfrey-Smith, 2016; Birch et al., 2021; Crook, 2021), arthropods (Mikhalevich & Powell, 2020), fishes, and decapod crustaceans (Gruen & Monsó, 2024).

These studies suggest that animals may be more susceptible to pain and suffering than previously recognized, indicating that current ethical standards and legal protections may require revision in light of these findings (Proctor, 2012; Kiani et al., 2022). This has led to increasing opposition to animal-based research from both scientists and the broader public (Kiani et al., 2022). As a result, the ethical treatment of animals in scientific studies has become a central concern in both biology and research ethics (Franco, 2013; Kiani et al., 2022).

The ethical discourse surrounding animal experimentation first gained momentum in 1959 with the introduction of the 3Rs framework: **R**eduction, **R**efinement, and **R**eplacement (Tannenbaum & Bennett, 2015; Mukherjee et al., 2022). This approach encourages researchers to limit the number of animals used to the minimum necessary for scientific validity and reliability (reduction), to minimize suffering or distress during procedures (refinement), and to seek non-animal alternatives whenever possible (replacement). These principles have long been viewed as the ethical cornerstone of laboratory animal use, yet questions remain about the consistency and rigor of their implementation in practice (Liguori et al., 2017; Mukherjee et al., 2022). In recent years, some scholars have proposed the addition of a fourth R: **R**esponsibility. This concept emphasizes the broader ethical obligation of researchers, not only to safeguard animal welfare but also to critically reflect on the societal and scientific justification for their experiments (Tannenbaum & Bennett, 2015; Kiani et al., 2022). As a result, many institutional animal ethics committees now evaluate proposed research through the lens of all four Rs, reinforcing a more comprehensive framework for ethical decision-making in animal studies (Lee et al., 2020; Kiani et al., 2022).

Ethical guidelines and legal protections have historically centered on vertebrates, especially mammals, due to their perceived similarity to humans and the more apparent signs of suffering they exhibit (Eddy et al., 1993; Mather, 2019; Kiani et al., 2022). This focus reflects a deep-rooted anthropocentric bias: humans are more inclined to empathize with animals that resemble us in terms of facial expressions, motor behaviors, cognitive complexity, and brain structure (Eddy et al., 1993; Broom, 2013; Jones, 2013; Mather, 2019). Consequently, mammals receive disproportionate attention in both policy and public discourse, despite accounting for only about 0.2% of the animal species diversity (Broom, 2013; Mather, 2019). In contrast, invertebrates, which enormously outnumber vertebrates in terms of absolute numbers and account for 90-99% of the species in the animal kingdom, are largely overlooked (Carere et al., 2011; Horvath et al., 2013; Mather, 2019; Iglesias, 2021). While public sentiment, media portrayals, and expanding research efforts continue to reinforce empathy toward mammals (Seddon et al., 2005; Sergio et al., 2008; Jones, 2013; Colléony et al., 2017), concern for “lower” animals remains rare (Horvath et al., 2013; Mather, 2019; Iglesias, 2021). The prevailing reluctance to engage with this subject appears rooted in the widespread perception that invertebrates lack traits that typically prompt human empathy, such as emotional expression, cognitive complexity, or neurological sophistication (Iglesias, 2021).

This neglect is further reinforced by public and scientific attitudes that often portray these organisms as “aliens” (Lockwood, 2013), leading to their exclusion from moral consideration and the frequent dismissal of their potential interests as insignificant (Iglesias, 2021). Building on this discourse, Mikhalevich and Powell (2020) challenge such assumptions by analyzing criteria commonly used to grant ethical consideration, namely consciousness, sentience, cognitive ability, emotional capacity, and pain perception in invertebrates (Howard & Symonds, 2020; Mikhalevich & Powell, 2020). They argue that invertebrates should be ethically regarded if they meet the same standards as vertebrates already protected under animal welfare regulations (Howard & Symonds, 2020; Mikhalevich & Powell, 2020). Nevertheless, invertebrates continue to be unfairly classified by the general public as inferior or simplistic organisms, often due to their smaller brain size and behaviors interpreted as purely instinctual (Howard & Symonds, 2020; Iglesias, 2021). This entrenched bias contributes to their persistent marginalization in ethical discussions, despite emerging evidence that calls for a reevaluation of their moral status (Howard & Symonds, 2020; Mikhalevich & Powell, 2020). If moral consideration is to be applied equitably, it is imperative to look beyond our instinctive preferences and extend ethical scrutiny to all sentient beings, not just those we find familiar or endearing (Mather, 2019).

One group of invertebrates that has become central to this ethical reconsideration is the cephalopods. While invertebrates are often dismissed because they lack emotional expression, cognitive complexity, or sophisticated nervous systems (Iglesias, 2021), cephalopods have increasingly stood out as a notable exception. A particular cephalopod species, first described by Georges Cuvier in 1797, and now extensively studied due to its ecological importance, behavioral complexity, and increasing application as a model organism in developmental and neurobiological research, is *Octopus vulgaris* (Crook 2021; Dominguez-Lopez et al., 2021; Almeida et al., 2022; Stern-Mentch, 2022; Styfhals et al., 2022; Dissegna et al., 2023; Papadopoulou et al., 2024). This benthic cephalopod species, also referred to as the common octopus, is widely distributed across temperate and tropical marine environments (MarineBio Conservation Society, n.d.; Amor et al., 2016; The Editors of Encyclopaedia Britannica, 2025). Following an embryonic period of approximately 30 days, *Octopus vulgaris* hatchlings enter a free-swimming, planktonic developmental phase known as the paralarval stage (Figure 1) (Iglesias et al., 2007; Roura et al., 2023). At hatching, these “paralarvae”—a term first introduced by Young and Harman (1988)—typically measure between 1.0 and 1.5 mm in mantle length, corresponding to a total size of approximately 1.5 to 2.9 mm (Iglesias et al., 2007). They swim actively in the water column and exhibit high metabolic rates, requiring large quantities of live prey with sufficient motility and nutritional quality to sustain rapid growth (Villanueva, 1995; Parra et al., 2000; Navarro & Villanueva, 2000, 2003; Iglesias et al., 2000, 2007). Throughout this planktonic phase, individuals undergo notable morphological changes, primarily driven by the disproportionate elongation of the arms relative to the mantle (Iglesias et al., 2007; Roura et al., 2023).

As a result, the squid-like form of newly hatched paralarvae progressively develops into the characteristic octopus body plan by the time of settlement (Iglesias et al., 2007; Roura et al., 2023). Similar to other developmental stages of *Octopus vulgaris*, the duration of this stage is temperature-dependent, typically lasting 47–54 days at 21.2 °C and 30–35 days at 23 °C (Villanueva, 1995; Iglesias et al., 2007). Once development reaches this point, individuals descend to the benthos, transitioning into the benthic juvenile phase (Iglesias et al., 2007; Roura et al., 2023). From there, they continue growing until they reach adulthood, typically within two years.

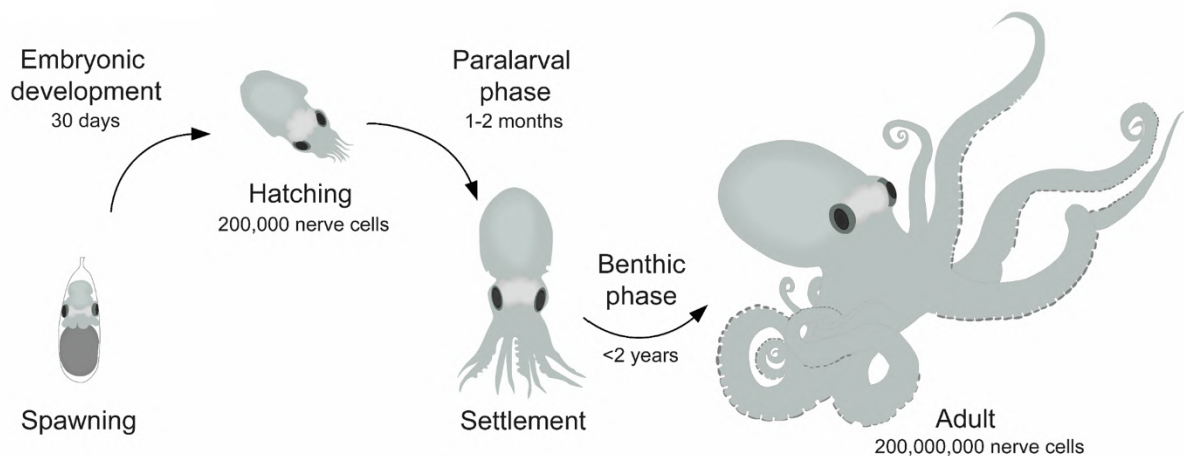


Figure 1: Life cycle of *Octopus vulgaris* and neural development across ontogeny (courtesy of Ruth Styfhals). During the spawning, *O. vulgaris* undergoes approximately 30 days of embryonic development before hatching with $\pm 200,000$ nerve cells. During the paralarval phase (1-2 months), the organism remains planktonic before settling to the benthos, marking the transition to the benthic phase. Within two years, individuals reach adulthood with an estimated 200 million nerve cells, reflecting dramatic post-embryonic neural development.

Scientific evidence now suggests that cephalopods, including *Octopus vulgaris*, possess advanced cognitive abilities, engage in complex behaviors, and are equipped with highly developed nervous systems (Mather & Dickel, 2017; Schnell et al., 2021; Ponte et al., 2021; Ponte et al., 2022; Albertin & Katz, 2023; Jozet-Alves et al., 2023; Shook et al., 2024). These traits have prompted researchers to investigate whether cephalopods can experience pain, using both neural and behavioral approaches (Andrews et al., 2013; della Rocca et al., 2015; Crook, 2021). A crucial consideration in this context is the distinction between nociception and pain, as both must be evaluated to determine whether pain is genuinely present in any given animal group (Elwood, 2011). Nociception refers to “the neural processes of encoding and processing noxious stimuli” (Loeser & Treede, 2008, p. 475) and is mediated by high-threshold sensory receptors of the peripheral somatosensory nervous system, known as nociceptors (IASP, 2021). While nociception is a necessary precondition for the possibility of pain, it is not sufficient on its own (Bateson, 1991; Broom, 2001; Elwood, 2011). A true pain experience involves conscious awareness of tissue damage and an associated negative emotional state (Elwood, 2011; IASP, 2021). In vertebrates, neural signals relating to tissue damage are not inherently “painful”; pain arises from the brain’s interpretation of these signals, shaped by awareness and affective context (Elwood, 2011). What is perceived is the damage itself; there is no direct perception of “pain” (Elwood, 2011). Instead, pain is the result of a strong, unpleasant emotional state that is closely linked to a motivation to end the experience triggered by those neural messages (Elwood, 2011).

For many, the idea that cephalopods, and invertebrates in general, could exhibit complex forms of consciousness is difficult to accept (Ponte et al., 2022). Yet, from a theoretical standpoint, our assumption that other humans are conscious is not based on direct access to their minds but rather on inference from shared behaviors and experiences, much like our belief in the reality of the external world (Humphrey, 2006; Andrews, 2020; Ponte et al., 2022). Following this logic, it becomes difficult to justify excluding all non-human animals from the realm of conscious beings (Ponte et al., 2022). It is therefore plausible that many animals, including cephalopods, possess a form of primary consciousness shaped by their own sensory and cognitive capacities, as well as by their anatomical features and environmental context (Feinberg & Mallatt, 2020; Ponte et al., 2022). Building upon this theoretical foundation, accumulating evidence now supports the view that cephalopods are sentient beings capable of experiencing pain (Andrews et al., 2013; della Rocca et al., 2015; Godfrey-Smith, 2016; Birch et al., 2021; Crook, 2021), a recognition that has begun to shape scientific policy and ethical frameworks. Reflecting this recognition, the European Union has extended the protective scope of Directive 2010/63/EU (2010) to include all “live cephalopods” used in scientific procedures that are likely to cause adverse effects, including pain, suffering, distress, or lasting harm (Smith et al., 2013; Fiorito et al., 2014). Because this protection applies to all “live cephalopods,” it also extends to larval stages, which fall under the legislation by virtue of being alive (Directive 2010/63/EU, 2010). However, although there is substantial evidence and growing consensus that adult cephalopods can experience pain, to the best of current knowledge, almost nothing is known about whether this also applies to their early developmental stages, including *Octopus vulgaris* paralarvae. This scientific uncertainty raises important ethical questions regarding the necessity and extent of regulatory protections at these stages.

To explore this issue, the present study investigates perceptions and reasoning on the ethical treatment of animals in octopus research, focusing on *O. vulgaris* paralarvae. Using an online survey, this study aims to explore differences in ethical judgments and underlying reasoning between scientific and non-scientific communities, and to assess whether exposure to different types of scientific background information influences these perspectives. To provide relevant scientific context for the survey, the thesis also includes a biological investigation into the expression of nociception-related genes in *O. vulgaris* paralarvae, an important first step in evaluating their potential for pain perception. While the identification of such genes may suggest the presence of nociceptors, it does not in itself confirm the capacity to experience pain. Nonetheless, these findings are valuable for future research. By addressing the complex interplay between biology, belief, and moral reasoning, this thesis aims to advance a more nuanced understanding of ethical judgment in scientific practice.

2. Objectives

Octopus vulgaris paralarvae remain a largely overlooked group in ethical debates surrounding animal research. Although limited empirical data exists on their sensory capacities, particularly regarding their ability to experience nociception or pain, they are nonetheless included under ethical protection frameworks. This scientific uncertainty raises important ethical questions regarding the necessity and extent of regulatory protections at this and other stages in the early development of cephalopods. Should early life stages be protected on the basis of potential sentience, even in the absence of conclusive data? Or should ethical treatment be reserved only for organisms whose capacity for pain has been clearly demonstrated? These questions give rise to deeper concerns about how and why we extend moral consideration to certain life stages or species. What drives different communities to regard such organisms as ethically relevant? What makes research on paralarvae ethically acceptable or unacceptable? And critically, how do these judgments differ between scientific and non-scientific communities? These questions form the basis of the present study, which aims to explore perceptions and reasoning on the ethical treatment of animals in octopus research across scientific and non-scientific communities. Additionally, it examines whether the provided scientific background information influences these ethical perceptions and the reasoning behind them.

To investigate this, an experimental philosophical study will be conducted using an online survey targeting three distinct participant groups: biologists working specifically with cephalopods, biologists working with other (model) organisms, and laypeople without a background in biology. Participants will first be asked to answer a set of demographic questions. Afterwards, they will be presented with a short abstract describing the outcome of a gene expression experiment, investigating the expression patterns of genes potentially linked to nociception in *Octopus vulgaris* paralarvae. Importantly, the information provided in this abstract will vary between participants; each participant will be randomly assigned to one of three versions. In Scenario 1, the gene expression experiment was performed, and nociception-related gene expression was observed in *O. vulgaris* paralarvae. In Scenario 2, the same experiment was conducted, but no expression of genes related to nociception was observed. In the final scenario, Scenario 3, the experiment had not yet been performed, and the presence or absence of genes related to nociception remains uncertain. After reading the abstract, participants will be asked to respond to a series of questions assessing their perceptions and reasoning regarding the use and ethical treatment of both *O. vulgaris* paralarvae and adults in scientific research. Prior to the study, several hypotheses were formulated and pre-registered to examine how perceptions and reasoning might vary across participant groups and experimental conditions. These hypotheses will be evaluated through statistical analysis of the survey responses, and the analyses will be conducted in R using the RStudio environment.

In addition to the ethical inquiry, a second aim of this thesis is to provide empirical context by analyzing the expression patterns of several genes related to nociception in *Octopus vulgaris* paralarvae. A total of six genes were selected for inclusion in the experiment based on their known or proposed roles in nociceptive pathways in adult *O. vulgaris*. Gene expression patterns will be assessed using hybridization chain reaction (HCR) in *O. vulgaris* paralarvae at 5 and 40 days post-hatching, corresponding to two distinct stages in the species' early development. While the presence of nociception-related genes does not in itself confirm the capacity for pain, it does provide important insights into sentience and potential pain perception in early developmental stages of cephalopods. Additionally, it provides contextual information on how nociceptive signals may differ between developmental stages.

While the primary focus of the thesis lies in exploring perceptions and reasoning of different communities regarding the ethical treatment of animals in octopus research, the inclusion of the biological data provides essential context. This thesis, in its entirety, contributes to ongoing debates in biology and animal research ethics by integrating empirical biological data with philosophical and ethical analysis. It provides insight into how scientific uncertainty and empirical framing influence ethical reasoning and sheds light on how disciplinary training and experience shape moral judgments. More broadly, it invites reflection on how we formulate ethical responsibilities in the face of incomplete knowledge, and how different communities interpret scientific evidence when making decisions about the treatment of sentient or potentially sentient organisms. To ensure clarity and coherence in the overall thesis structure, the biological component is presented separately as an attached "Microthesis," beginning on page 31. Presenting this section as a distinct part allows for a more focused and structured discussion of the gene expression study, emphasizing its scientific relevance without disrupting the main thesis narrative.

3. Materials and Methods

This study was approved by the Social and Societal Ethics Committee of KU Leuven (SMEC), Belgium (file no. G-2025-9207-R2(MIN)). Data collection only started after the full research design was pre-registered on the Open Science Framework (OSF) (Monnissen & de Block, 2025). Only individuals aged 18 years or older were eligible to participate in the study, and participation was entirely voluntary. To safeguard participants' privacy, all responses were handled with the utmost care and confidentiality. Additionally, all data presented in this thesis is fully anonymized to ensure that no individual can be identified.

3.1. Design

A detailed empirical investigation into perceptions and reasoning on the ethical treatment of animals in octopus research was conducted using an online survey administered through LimeSurvey (LimeSurvey GmbH, n.d.). The questionnaire was developed by the student researcher and author of this thesis, Jill Monnissen, and refined following feedback from Prof. Dr. Andreas De Block, the thesis supervisor. To capture relevant background information, participants were first asked to self-identify as belonging to one of three groups: (1) biologists working with cephalopods (hereafter referred to as “cephalopod biologists”), (2) biologists working with other (model) organisms (hereafter referred to as “other biologists”), and (3) non-biologists. Following this classification, demographic information was collected from all participants, including their age, nationality, and gender. Additionally, non-biologists were exclusively asked to indicate their highest level of education. In contrast, biological respondents were requested to specify their field of expertise, level of academic experience (less than 5 years, 5–10 years, 11–15 years, 16–20 years, or more than 20 years), the (model) organisms they have worked with, and whether they have conducted or supervised research involving *O. vulgaris*. All participants were also asked to rate their familiarity with research ethics on a scale from 1 (not at all familiar) to 5 (very familiar). After completing these background questions, participants were presented with a short abstract describing the outcome of a gene expression experiment investigating the presence of nociception-related genes in *O. vulgaris* paralarvae. To allow for comparison between different informational conditions, participants were randomly assigned to one of three versions of the abstract. In the first scenario, the abstract reported that the experiment had been conducted, and expression of genes related to nociception could be observed. In the second scenario, the experiment was also conducted, but there was no expression of the nociception-related genes. The third, and final, version of the abstract stated that the experiment had not yet been conducted, thereby introducing a context of scientific uncertainty. This experimental manipulation enabled the assessment of how different types of empirical context—evidence for nociception, no evidence for nociception, or unresolved uncertainty—shaped participants' ethical judgments.

Importantly, the information about the gene expression experiment presented in the abstract was not entirely hypothetical—the vignettes weren't all mock abstracts. After all, the vignettes were based on an actual experiment conducted as part of the biological component of this thesis. Using hybridization chain reaction (HCR), the expression patterns of six genes potentially related to nociception were investigated in *O. vulgaris* paralarvae. The experimental results confirmed that these genes were indeed expressed, corresponding to the outcome described in the first scenario. The second and third scenario, which reported the absence of gene expression or unresolved uncertainty, were constructed using the same experimental data framework but were intentionally altered to serve the purposes of experimental manipulation within the survey. To maintain transparency and avoid the transmission of misinformation, all participants were debriefed at the end of the questionnaire and informed about the actual results of the experiment. A detailed overview of the biological experiment is provided in the attached “Microthesis” starting on page 31.

After reading their assigned version of the abstract, participants were asked to respond to a series of questions evaluating their ethical judgments concerning scientific research on both *Octopus vulgaris* paralarvae and adults. First, participants were presented with the full set of questions focusing exclusively on the paralarval stage. Once completed, the same questions were repeated for the adult stage. Participants were asked whether they believed the animals were capable of experiencing pain and how confident they were in this judgment. They were also asked whether additional research was necessary to resolve the uncertainty surrounding pain perception in *O. vulgaris*. If participants answered affirmatively, they were prompted to indicate what type of further research they believed was needed. Additional questions assessed participants' views on the ethical acceptability of scientific research, its societal relevance, and the extent to which they agreed with the notion that the societal relevance of this research outweighs the potential pain experienced by *O. vulgaris* individuals. To maintain clarity in the main text, specific answer formats (e.g., yes/no options, Likert scales) are not detailed here. A complete overview of all survey questions and response options is provided in Section 8 (Addendum, pages A.1 – A.13).

3.2. Sampling

The survey was distributed via email, professional networks, and the personal social media platforms of the student researcher, including Facebook, Instagram, and LinkedIn. Due to the use of a snowball sampling strategy, it was not possible to calculate a precise response rate or determine how representative the sample is of the broader population, a common limitation in this type of survey-based research. Data collection commenced on March 26th and was closed on April 14th, as pre-registered on OSF (Monnissen & de Block, 2025).

The survey primarily targeted two broad groups: biologists and laypeople without formal biological training (non-biologists). Within the biologist group, a further distinction was made between those working specifically with cephalopods and those working with other (model) organisms. A total of 294 individuals participated in the survey. For the final analysis, only fully completed responses were retained, resulting in a total sample size of 205 participants. Prior to data collection, a minimum target of 50 responses per group was established. This was successfully achieved, with a total of 52 responses from cephalopod biologists, 94 from other biologists, and 59 from non-biologists.

To ensure participants' responses were not compromised by poor English proficiency, participants were asked to self-rate their proficiency on a scale from 1 (very poor) to 5 (excellent). Scores of 3 and higher were regarded as sufficient for understanding the survey content. No participant rated their proficiency below 3, except for one individual who indicated a score of 1. As this was an isolated case and unlikely to significantly affect the overall results, the response was retained in order to preserve the sample size and maintain statistical power.

3.3. Hypotheses and Statistical Analyses

To investigate how empirical information, moral beliefs, and personal background shape ethical evaluations of animal research, a total of seven hypotheses were formulated and pre-registered on the Open Science Framework (OSF) (Monnissen & de Block, 2025). These hypotheses were informed by assumptions found in the literature as well as by the interpretations of both the student researcher and the project supervisor. They specifically focus on *Octopus vulgaris* paralarvae, which constitute the central subject of this study.

The first two hypotheses focus on what might lead participants to consider research on *O. vulgaris* paralarvae as “unethical.” It is anticipated (Hypothesis 1) that participants exposed to the scenario in which nociception-related genes are expressed (Scenario 1) will be less likely to consider invasive or pain-inducing research on *O. vulgaris* paralarvae ethically acceptable, compared to participants exposed to the scenario where such genes are not expressed (Scenario 2) and those exposed to scientific uncertainty (Scenario 3). The expectation here is that evidence of nociception-related gene expression may lead participants to infer that paralarvae can feel pain, increasing their ethical concern. In contrast, the absence of such evidence or the presence of uncertainty offers less ground for concern, potentially making the research seem more acceptable. Building on this line of reasoning, Hypothesis 2 proposes that participants who believe further research is necessary to resolve uncertainty about pain in *O. vulgaris* paralarvae will be more likely to consider invasive or pain-inducing research as “unethical,” compared to those who do not believe such research is necessary. Individuals who support further investigation may already suspect that paralarvae can experience pain and therefore view resolving this uncertainty as especially important. As a result, they may approach the ethics of current research more cautiously.

The next three hypotheses investigate participants' views on whether the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. First, it is expected (Hypothesis 3) that participants who believe *O. vulgaris* paralarvae are capable of experiencing pain will be less likely to agree that the societal relevance of the research outweighs this pain, compared to those who do not hold this belief. When participants acknowledge the sentience of the animal, the moral weight of inflicting pain likely increases, making it harder to justify. Those who do not believe the animals can feel pain may, on the other hand, be more inclined to prioritize the potential societal benefits. Additionally, it is hypothesized (Hypothesis 4) that participants who report greater self-rated familiarity with animal research ethics will assign higher importance to societal relevance outweighing potential pain, compared to those with lower levels of familiarity. Familiarity with ethical frameworks may lead these individuals to be more accustomed to the trade-offs commonly encountered in research, making them more willing to accept causing pain when it serves a broader societal purpose. Those with less familiarity might approach such trade-offs with more hesitation. Furthermore, it is anticipated (Hypothesis 5) that participants exposed to the scenario in which nociception-related genes are expressed will be less likely to agree that societal relevance outweighs potential pain, compared to participants in the scenarios without gene expression and with scientific uncertainty. Exposure to scientific evidence indicating a capacity for nociception may heighten concern about the moral cost of inflicting pain, making societal justifications feel less adequate. Where no expression is found or uncertainty remains, participants may perceive less ethical risk and may therefore be more inclined to accept that the societal relevance of the research outweighs the potential for pain.

The perceived societal relevance of the research may also influence other judgments. Hypothesis 6 proposes that participants who rate the societal relevance of the research as "high" will be less likely to support the need for further research to resolve uncertainty about pain experience in *O. vulgaris* paralarvae, compared to those who rate the societal relevance as "low." Participants who perceive the research as especially relevant to society may be more inclined to proceed despite unresolved ethical concerns, perhaps because they prioritize societal benefits over resolving uncertainty about pain. In contrast, those who consider the research less societally relevant may be more cautious and likely to support further research.

Finally, Hypothesis 7 addresses confidence in pain interpretation. It is expected that participants with more years of academic experience in scientific research, especially those with formal biological training, will report higher levels of confidence in evaluating the possibility of pain in *O. vulgaris* paralarvae, compared to those with less and those with no experience. Their background likely equips them to interpret scientific information with greater ease and assurance. In contrast, those with no or low experience may feel less qualified to assess the issue, resulting in lower confidence.

Alongside the seven pre-registered hypotheses, two additional hypotheses were tested to further examine participants' beliefs about paralarval pain. First, it was hypothesized that participants exposed to the gene expression scenario would be more likely to believe that paralarval *O. vulgaris* are capable of experiencing pain, compared to those exposed to the other two conditions. Scientific evidence for nociception may strongly influence beliefs, whereas the absence of evidence or the presence of uncertainty may leave participants less convinced. Second, it was anticipated that cephalopod biologists would be more likely to attribute the capacity to feel pain to paralarvae compared to other biologists and non-biologists. Their specialized expertise and familiarity with *O. vulgaris* likely make them more attuned to recent scientific developments related to sentience and pain, whereas participants without such background knowledge may be less inclined to draw this conclusion.

Statistical analyses and figure generation were conducted in R v4.3.1 (R Core Team, 2023), within the RStudio v2023.06.0+421 environment. Proportions were used in the final analyses to allow for meaningful comparisons between groups of different sizes. To ensure accurate estimation of the overall effects across factor levels, sum contrasts were applied using the `set_sum_contrasts()` function from the `afex` package (Singmann et al., 2023). Logistic regression was used for hypothesis testing, with model development incorporating not only the core predictor and outcome variables but also a consistent set of background variables, including *Scenario*, *Group*, *Age*, *Sex*, *Nationality*, and *RE*. *Scenario* refers to the type of scientific background information participants were exposed to, including gene expression, no gene expression, or unresolved uncertainty; *Group* distinguishes between cephalopod biologists, other biologists, and non-biologists; *Age* indicates the participant's age group in years, categorized as 18–30, 31–40, 41–50, or 50 and above; *Sex* distinguishes between male, female, and non-binary individuals; *Nationality* refers to the participant's country of origin; and *RE* reflects participants' self-rated familiarity with research ethics, measured on a 5-point Likert scale. For analysis, *RE* was recoded into a binary variable with scores of 1 to 3 being classified as “low familiarity” (*RE_Low*), and scores of 4 and 5 as “high familiarity” (*RE_High*). The most appropriate model for each hypothesis was determined using `glmulti` from the `glmulti` package v1.0.8 (Calcagno, 2020). This facilitated automated model selection based on information-theoretic criteria. Once the optimal model was identified, logistic regression analysis was performed. To assess overall significance, a type III Anova was conducted using the `Anova` function from the `car` package v3.2.1 (Fox & Weisberg, 2019). Where relevant, pairwise comparisons were performed using the `emmeans` function from the `emmeans` package v1.9.0 (Lenth, 2023). For the comparisons, Bonferroni correction was used to reduce the risk of false positives, which is especially important in the context of unequal group sizes. To provide clear and informative visualizations of the results, figures were created using the `ggplot` function from the `ggplot2` package v3.4.4

(Wickham, 2016).

4. Results

4.1. What Influences Ethical Judgment?

This section examines what causes participants to be more or less likely to rate research performed on *O. vulgaris* paralarvae as “unethical.” To explore this, two hypotheses were formulated and tested using logistic regression. The response variable of this analysis represented the proportion of participants rating the research as “unethical.” During the survey, participants were asked to rate how ethical they found the research on a scale from 1 to 5. Scores of 3 and lower were categorized as “unethical” (*Ethical_Low*), while scores of 4 and 5 were indicated as “ethical” (*Ethical_High*).

The first hypothesis (Hypothesis 1) focused on the effect of the scientific background information (*Scenario*) participants received during the survey. It was predicted that participants exposed to the scenario in which nociception-related genes were expressed (Scenario 1) would be more likely to rate invasive or potentially pain-inducing research on *O. vulgaris* paralarvae as “unethical,” compared to those exposed to the scenario in which no gene expression was observed (Scenario 2) and the scenario indicating unresolved uncertainty (Scenario 3). The second hypothesis (Hypothesis 2) considered the participants’ belief about whether further research is necessary to resolve uncertainty about pain in *O. vulgaris* paralarvae. It was predicted that participants who believe further research is necessary would be more likely to rate the research as “unethical,” compared to those who do not believe further research is needed. During the survey, participants were asked: “Do you believe more research is necessary to resolve this uncertainty concerning pain experience in *O. vulgaris* paralarvae?” They could respond with “Yes,” “No,” or “I don’t believe there is uncertainty.” For this analysis, this variable (*More_res_p*) was recoded as a binary outcome, with “Yes” responses indicating participants believed more research was needed (*MoreRes_Yes*), and “No” and “I don’t believe there is uncertainty” grouped together to reflect participants who did not believe further research was necessary (*MoreRes_No*).

A logistic regression analysis was conducted to assess these hypotheses. The best model, as identified by *glmulti*, included the main effects of *Group* and *RE_High*. *Group* distinguishes between cephalopod biologists, other biologists, and non-biologists, while *RE_High* represents participants’ familiarity with animal research ethics as either “high” or “low.” Notably, the variables *Scenario* and *MoreRes_Yes*, which are essential for testing the two hypotheses described above, were not selected as part of the best model. However, given their theoretical importance, both variables were manually added to evaluate their individual effects and draw conclusions regarding the hypotheses.

The logistic regression analyses revealed that the proportion of participants rating the research as “unethical” did not differ significantly across scenarios (Figure 2). Participants exposed to the scenario in which nociception-related gene expression was observed (Scenario 1) were not significantly more likely to rate the research as “unethical,” compared to those exposed to Scenario 2 ($p = 1$), where no gene expression was observed, and Scenario 3 ($p = 1$), which indicated unresolved uncertainty. Additionally, there was also no significant difference observed in the proportion of participants rating the research as “unethical” between Scenario 2 and Scenario 3 ($p = 1$). These findings do not support Hypothesis 1 and suggest that participants’ ethical evaluations were not influenced by the type of scientific background information they received.

Furthermore, participants who indicated that further research is needed to resolve uncertainty about pain in *O. vulgaris* paralarvae were not significantly more likely to rate the research as “unethical,” compared to those who did not believe further research is necessary ($p = 0.50$). These findings are visualized in Figure 3 and suggest that Hypothesis 2 is not supported.

Finally, even though self-rated familiarity with research ethics (*RE_High*) was part of the best model, this did not significantly affect participants’ ethical judgments ($p = 0.16$). There was no significant difference in the proportion of participants rating the research as “unethical” between those who reported high familiarity with research ethics and those who reported low familiarity.

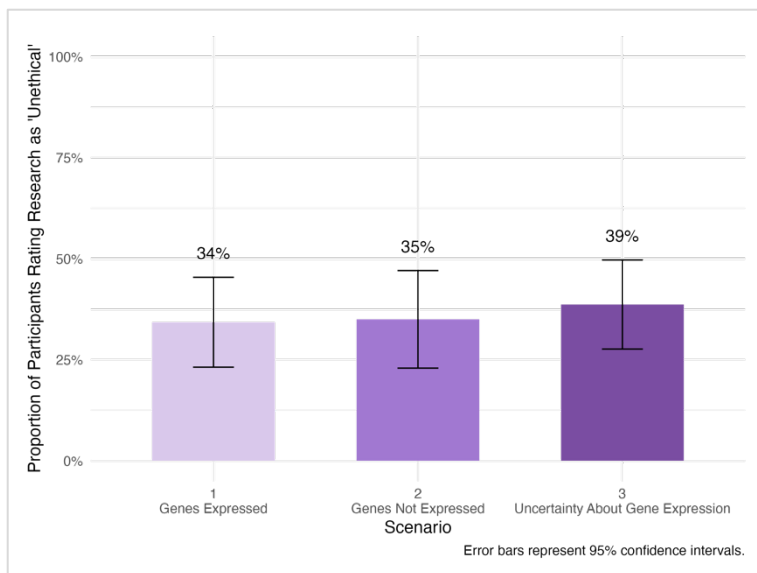


Figure 2: Effect of the received scientific background information (Scenario) on the proportion of participants rating the research on *O. vulgaris* paralarvae as “unethical.” The proportion of participants rating the research as “unethical” is equal to 34% for Scenario 1 (genes expressed), 35% for Scenario 2 (genes not expressed), and 39% for Scenario 3 (uncertainty about gene expression). There is no significant difference in the proportion of participants rating the research as “unethical” across the three scenarios. Error bars represent 95% confidence intervals.

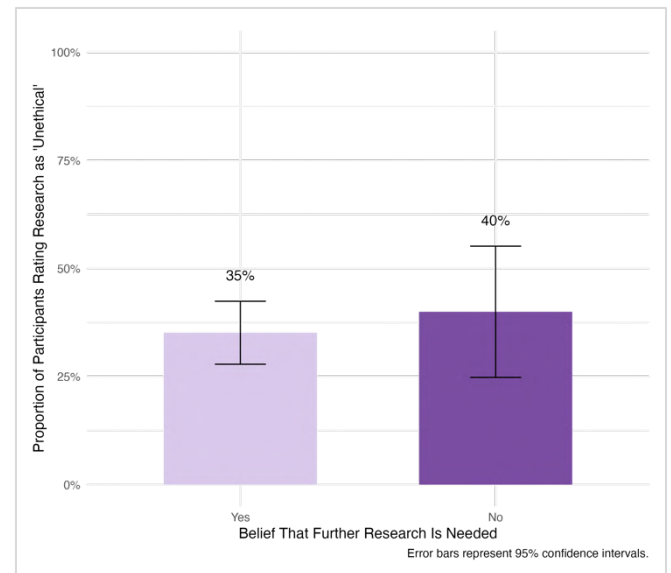


Figure 3: Effect of the belief in further research on the proportion of participants rating the research on *O. vulgaris* paralarvae as “unethical.” Among participants who believed that further research is needed (“Yes”), 35% rated the research as “unethical.” This was not significantly different from participants who indicated that no further research is needed (“No”), of whom 40% rated the research as “unethical.” Error bars represent 95% confidence intervals.

There was, however, a significant effect of the predictor variable *Group* ($p = 0.027$; $*p \leq 0.05$). Specifically, non-biologists were significantly more likely to rate the research as “unethical,” compared to cephalopod biologists ($p = 0.027$; $*p \leq 0.05$). No significant differences were observed between the other biologists and either of the remaining groups. These findings are illustrated in Figure 4.

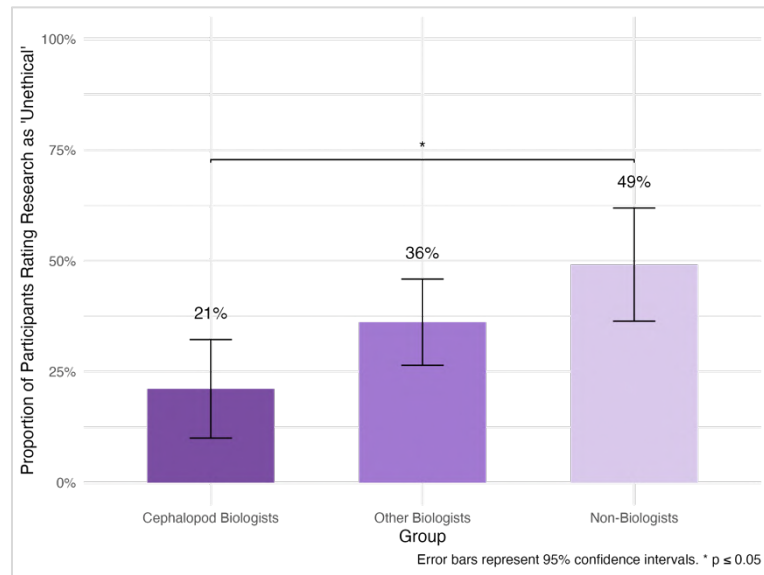


Figure 4: Effect of the participant group on the proportion of participants rating the research on *O. vulgaris* paralarvae as “unethical.” The proportion of participants rating the research as “unethical” is equal to 21% for the cephalopod biologists, 36% for the other biologists, and 49% for the non-biologists. There is a significant difference between the cephalopod biologists and non-biologists ($*p \leq 0.05$), with non-biologists being significantly more likely to rate the research as “unethical,” compared to cephalopod biologists. No other significant differences were observed. Error bars represent 95% confidence intervals.

4.2. What Influences Agreement With Societal Relevance Outweighing Pain?

This section takes a closer look at what causes participants to be more or less likely to agree that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. To explore this, three hypotheses were formulated and tested using logistic regression. The response variable of this analysis represented the proportion of participants agreeing that the societal relevance of the research outweighs the potential pain. During the survey, participants were asked to what extent (scale from 1 to 5) they agree with the statement “The societal relevance of this research ethically outweighs the potential pain experienced by paralarval *O. vulgaris*.” To facilitate the analysis, this response variable (*Soc_eth_p*) was recoded as a binary response with scores of 3 and lower being indicated as “participants disagreeing” (*SocEth_Low*), and scores of 4 and 5 as “participants agreeing” (*SocEth_High*).

The first hypothesis of this section (Hypothesis 3) predicted that participants who believed *O. vulgaris* paralarvae are capable of experiencing pain would be less likely to agree that the societal relevance of the research outweighs this pain, compared to participants who do not hold this belief. The binary predictor variable *P_pain* represented the participants’ beliefs of pain experience in *O. vulgaris* paralarvae. This variable was constructed based on participants’ responses (“Yes” or “No”) to the question of whether they believed paralarvae are capable of experiencing pain.

The second hypothesis (Hypothesis 4) predicted that participants with high self-rated familiarity with research ethics will be more likely to agree that the societal relevance of the research outweighs potential pain experienced by *O. vulgaris* paralarvae, compared to participants with low self-rated familiarity. Finally, the third hypothesis (Hypothesis 5) predicted that participants exposed to the scenario in which nociception-related gene expression was observed (Scenario 1) would be less likely to agree that the societal relevance outweighs potential pain, compared to those exposed to the scenario in which no gene expression was observed (Scenario 2) and the scenario indicating unresolved uncertainty (Scenario 3). A logistic regression analysis was conducted to assess these three hypotheses described above. The best model, as identified by glmulti, included the main effects of *Sex* and *P_pain*, and the interaction effect of *Scenario* and *Group* (*Scenario:Group*). Notably, the main effects of *Scenario* and *RE_High*, which are essential for testing Hypothesis 4 and Hypothesis 5, were not selected as part of the best model. However, given their theoretical importance, both variables were manually added to evaluate their individual effects and draw conclusions regarding the hypotheses.

The regression analysis revealed there was no significant effect of the interaction between *Group* and *Scenario* ($p = 0.16$) on the proportion of participants agreeing that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. The main effect of *Sex*, however, did have a significant effect ($p = 0.012$; $*p \leq 0.05$). As illustrated in Figure 5, male participants were significantly more likely to agree that societal relevance outweighs the potential pain experienced by *O. vulgaris* paralarvae, compared to female participants ($p = 0.0084$; $**p \leq 0.01$). Due to the small number of non-binary participants, this group was excluded from the interpretation.

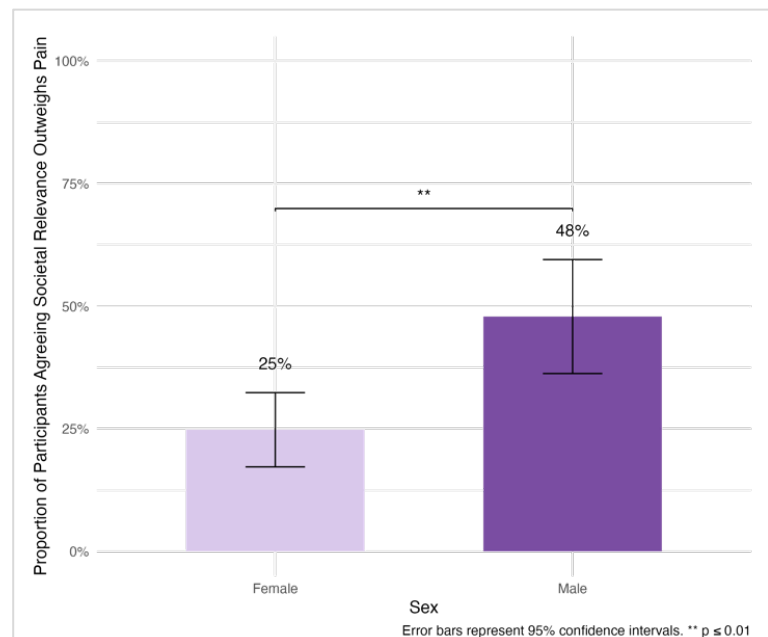


Figure 5: Effect of participants' gender (Sex) on the proportion of participants agreeing that the societal relevance of the research outweighs potential pain experienced by *O. vulgaris* paralarvae. Only 25% of all female participants agreed that the societal relevance outweighs the potential pain experienced by *O. vulgaris* paralarvae. Male participants, on the other hand, were significantly more likely ($**p \leq 0.01$) to agree that societal relevance outweighs potential pain, with 48% of all males agreeing to this. Non-binary participants are excluded from the interpretation due to their small sample size. Error bars represent 95% confidence intervals.

In addition, belief in paralarval pain (P_{pain}) also showed a significant effect. Specifically, participants who believed that *O. vulgaris* paralarvae are capable of experiencing pain were significantly less likely to agree that the societal relevance of the research outweighs this pain ($p = 0.014$; $*p \leq 0.05$), compared to those who did not believe paralarvae are capable of experiencing pain (Figure 6). This finding aligns with the prediction made in Hypothesis 3, and therefore, this hypothesis is supported.

Furthermore, the regression analysis revealed no significant effect of familiarity with research ethics (RE_High) on the proportion of participants who agreed that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. Contrary to the prediction in Hypothesis 4, participants with high familiarity were not significantly more likely to agree with this trade-off, compared to those with low familiarity ($p = 0.14$). Therefore, Hypothesis 4 is not supported. This result is illustrated in Figure 7.

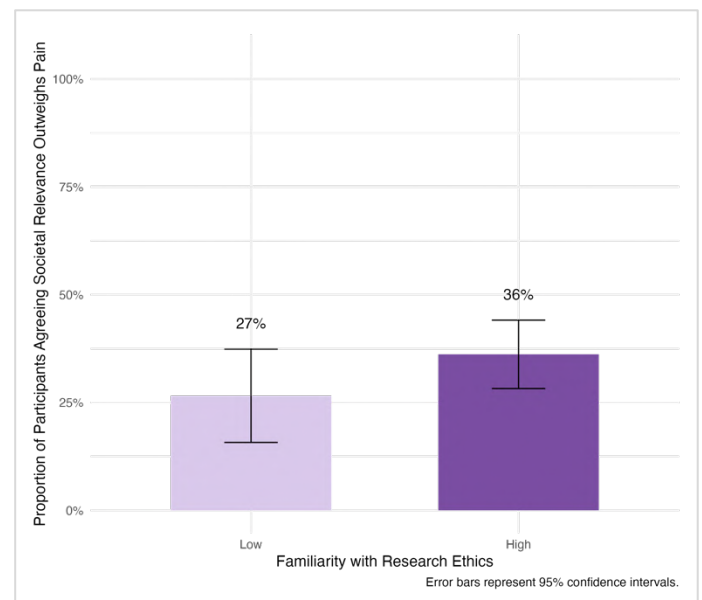
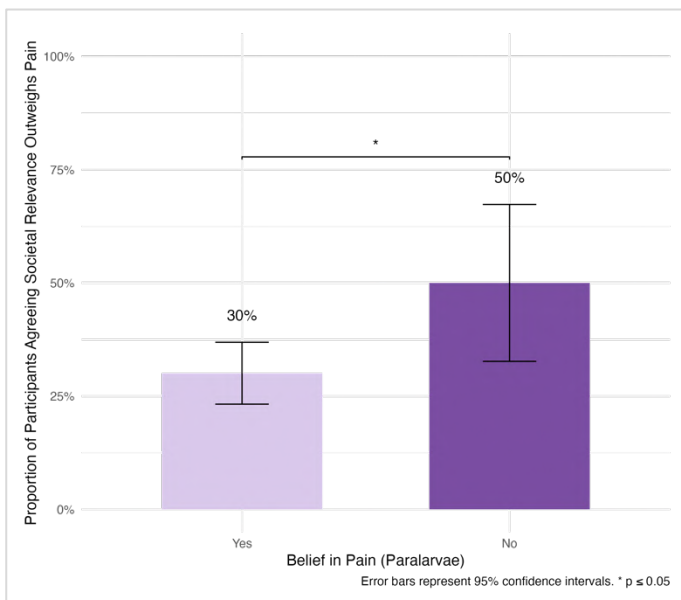


Figure 6: Effect of belief in paralarval pain on the proportion of participants agreeing that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. 30% of the participants who believed that *O. vulgaris* paralarvae are capable of experiencing pain agreed that societal relevance outweighs this pain. This percentage was significantly higher ($*p \leq 0.05$) for participants who do not believe paralarvae are capable of experiencing pain. In this last group, 50% of participants agreed that the societal relevance of the research outweighs potential pain experienced by the paralarvae. Error bars represent 95% confidence intervals.

Figure 7: Effect of familiarity with research ethics on the proportion of participants agreeing that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. Among participants who reported high familiarity with research ethics, 38% agreed that the societal relevance outweighs potential pain. In contrast, only 27% of participants with low familiarity agreed with this trade-off. However, this difference was not statistically significant. Error bars represent 95% confidence intervals.

Finally, the analysis revealed there was also no significant effect of the received scientific background information (*Scenario*) on the proportion of participants agreeing that societal relevance outweighs the potential pain experienced by *O. vulgaris* paralarvae ($p = 0.35$). As visualized in Figure 8, participants exposed to Scenario 1 were not significantly more likely to agree with this trade-off, compared to those exposed to Scenario 2 ($p = 0.60$) and Scenario 3 ($p = 1$). There was also no significant difference between participants exposed to Scenario 2 and Scenario 3 ($p = 0.62$). Therefore, it can be concluded that Hypothesis 5 is also not supported.

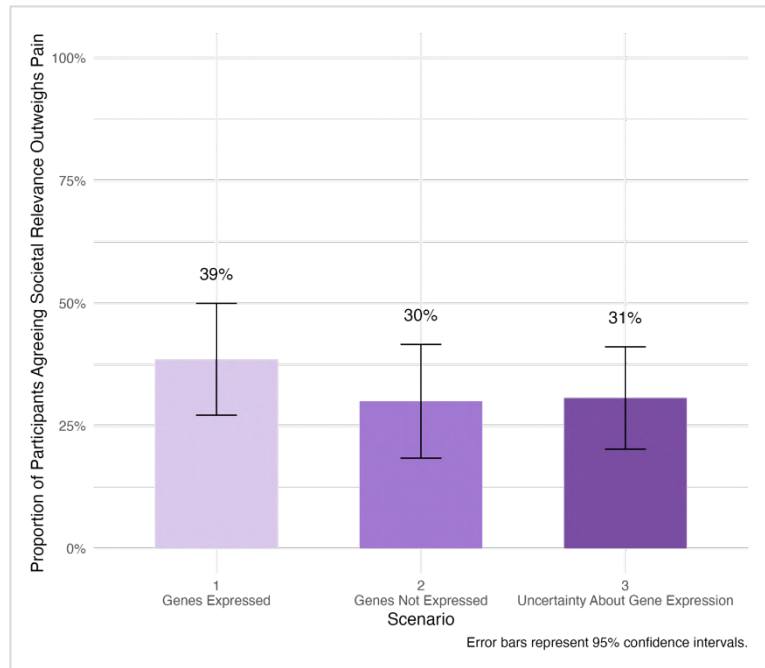


Figure 8: Effect of the received scientific background information (*Scenario*) on the proportion of participants agreeing that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae. The proportion of participants agreeing that societal relevance outweighs potential pain is equal to 39% for Scenario 1 (genes expressed), 30% for Scenario 2 (genes not expressed), and 31% for Scenario 3 (uncertainty about gene expression). There is no significant difference observed across the three scenarios. Error bars represent 95% confidence intervals.

4.3. What Influences Belief in the Need for Further Research?

Hypothesis 6 predicted that participants who rated the societal relevance of the research as “high” would be less likely to believe that further research is necessary to resolve uncertainty about pain in *O. vulgaris* paralarvae, compared to those who rated societal relevance as “low.” During the survey, participants were asked to rate the societal relevance of the research on *O. vulgaris* paralarvae on a scale from 1 to 5. However, for the analysis, this variable (*Soc_rel_p*) was recoded into a binary outcome with scores of 3 and lower being classified as “low” societal relevance (*SocRel_Low*), and scores of 4 and 5 as “high” societal relevance (*SocRel_High*). A logistic regression analysis was conducted to assess the hypothesis. The response variable used for this analysis represented the proportion of participants saying that further research is needed (*MoreRes_Yes*). More details about this variable can be found in Section 4.1 (pages 13 – 15).

The best model, as identified by glmulti, included the main effects of *Scenario* and *Sex*, as well as their interaction effect *Scenario:Sex*. This model did not include *SocRel_High*, suggesting that societal relevance was not among the strongest predictors of whether participants believed further research was necessary. Nonetheless, *SocRel_High* was manually added to examine its effect and formally test the hypothesis.

The regression analysis revealed no significant effect of the societal relevance (*SocRel_High*) on the proportion of participants saying further research is necessary. Specifically, participants who rated the societal relevance of the research as “high” were not significantly less likely to state that further research is needed, compared to participants who rated the societal relevance as “low” ($p = 0.72$). Therefore, Hypothesis 6 is not supported. These findings are illustrated in Figure 9. Additionally, while neither *Scenario* nor its interaction with *Sex* (*Scenario:Sex*) had a significant effect, the analysis did reveal a significant effect of the main effect *Sex* ($p = 0.0075$; $**p \leq 0.01$). As visualized in Figure 10, male participants were significantly less likely to indicate that further research is needed, compared to female participants ($p = 0.043$; $*p \leq 0.05$).

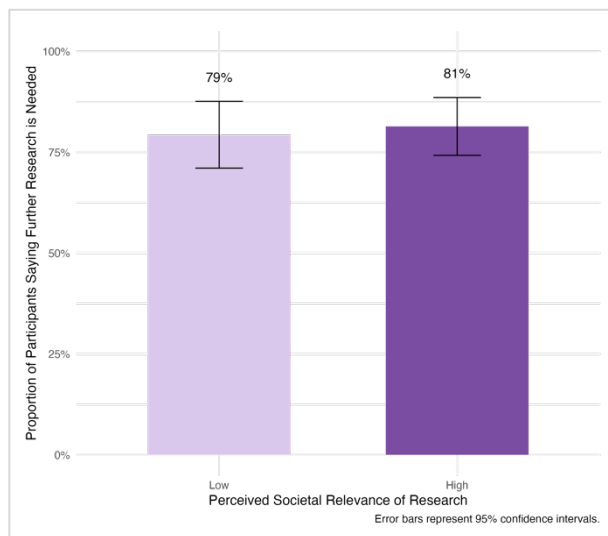


Figure 9: Effect of the perceived societal relevance (high vs low) on the proportion of participants saying that further research is needed to resolve uncertainty about pain in *O. vulgaris* paralarvae. Among participants who perceived the societal relevance of the research as “low,” 79% indicated that more research is needed to resolve uncertainty about pain in *O. vulgaris* paralarvae. This proportion did not differ significantly from those who perceived the societal relevance of the research as “high,” of whom 81% indicated more research is needed. Error bars represent 95% confidence intervals.

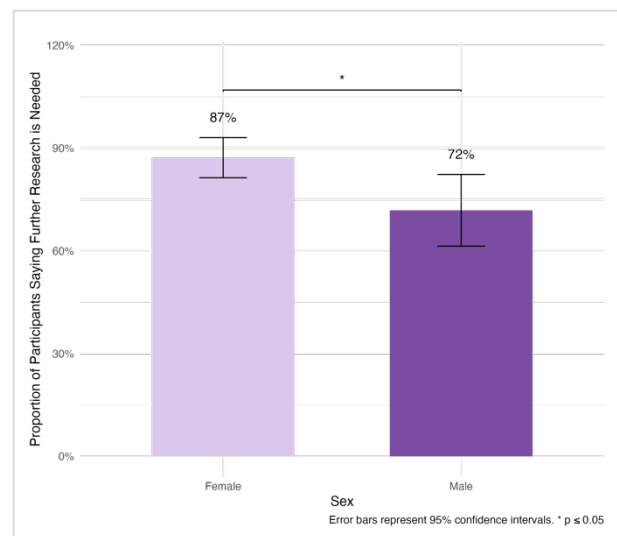


Figure 10: Effect of participants’ gender (Sex) on the proportion of participants saying that further research is needed to resolve uncertainty about pain in *O. vulgaris* paralarvae. Male participants were significantly less likely to indicate that further research is needed, compared to female participants ($*p \leq 0.05$). Specifically, 87% of female participants stated that further research is needed, compared to only 72% of male participants. Non-binary participants were excluded from the interpretation due to their small sample size. Error bars represent 95% confidence intervals.

4.4. What Influences Confidence in Pain Interpretation?

Finally, Hypothesis 7 predicted that participants with more years of academic experience would be more likely to report high confidence in evaluating the possibility of pain in *O. vulgaris* paralarvae, compared to those with less and those with no experience. A logistic regression analysis was conducted to assess the hypothesis. The response variable represented the proportion of participants being highly confident in their interpretation of paralarval pain. During the survey, participants were asked to rate their confidence in interpreting paralarval pain experience on a scale from 1 to 5. For the analysis, this response variable (*Conf_p_pain*) was recoded as a binary outcome with scores of 3 and lower indicating “low confidence” (*Conf_Low*), and scores of 4 and 5 indicating “high confidence” (*Conf_High*). The predictor variable for academic experience (*AE*) originally contained five levels, indicating the years of experience as 0, less than 5, 5-10, 11-15, 16-20, and more than 20. However, for the analysis, this variable was recoded to contain just three levels, indicating the academic experience to be “none” (*AE* = 0), “low” (*AE* = <5, 5-10, 11-15), or “high” (*AE* = 16-20, 20+).

The best model, as identified by glmulti, included *AE*, *RE_High*, and several interaction effects, including *Scenario:Sex*, *Scenario:Group*, and *RE_High:Group*. The logistic regression analysis revealed that only academic experience had a significant effect on the proportion of participants who reported being highly confident in their interpretation of paralarval pain (Figure 11). More specifically, participants with high academic experience were significantly more likely to report high confidence in their interpretation of pain in *O. vulgaris* paralarvae, compared to those with low academic experience ($p = 0.025$; $*p \leq 0.05$), thereby supporting the hypothesis.

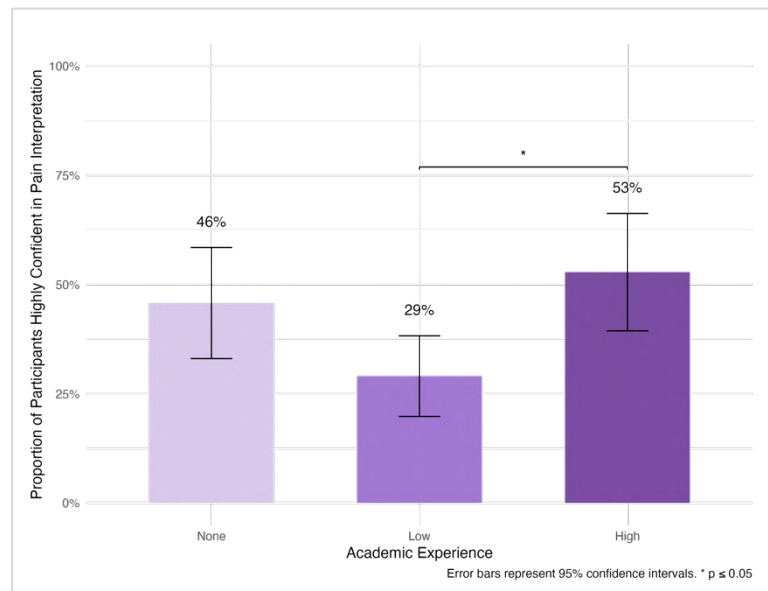


Figure 11: Effect of academic experience (“none,” “low,” or “high”) on the proportion of participants reporting “high confidence” in their interpretation of pain in *O. vulgaris* paralarvae. The highest proportion of participants indicating high confidence in their interpretation of paralarval pain was observed in the group with the most academic experience, of whom 53% reported being highly confident. This proportion was significantly higher ($*p \leq 0.05$) than that of participants with low academic experience, of whom only 29% reported being highly confident. Among participants with no academic experience, 48% reported high confidence, which did not differ significantly from either the high or low academic experience groups. Error bars represent 95% confidence intervals.

However, the model showed no significant differences in confidence ratings between participants with no academic experience and those with low experience ($p = 0.85$), nor between participants with no experience and those with high experience ($p = 1$). This suggests that participants without any academic experience in scientific research were not significantly more or less confident than those with some or extensive experience, thereby not supporting this last hypothesis. Thus, overall, this last hypothesis is only partially supported. While participants with high academic experience were significantly more likely to report high confidence, compared to participants with low academic experience, this was not the case compared to participants with no academic experience.

4.5. What Influences Belief in Paralarval Pain Experience?

Two additional, not previously registered, hypotheses were tested to examine which factors might influence participants' beliefs about pain experience in *O. vulgaris* paralarvae. The binary variable *P_pain* captured these beliefs and was constructed based on participants' responses ("Yes" or "No") to the question of whether they believed paralarvae are capable of experiencing pain. The first additional hypothesis predicted that participants exposed to the scenario in which nociception-related gene expression was observed (Scenario 1) would be more likely to believe that paralarvae are capable of experiencing pain, compared to those exposed to the scenario in which no gene expression was observed (Scenario 2) and the scenario indicating unresolved uncertainty (Scenario 3). Furthermore, the second additional hypothesis predicted that cephalopod biologists would be more likely to believe in paralarval pain experience, compared to other biologists and non-biologists.

Logistic regression was used to test both hypotheses, with the proportion of participants believing paralarvae were capable of experiencing pain (*P_Pain_Yes*) as the response. The best model was identified, using *glmulti*, and included *Scenario* and *RE_High* as predictors. The variable *Group* was not selected as part of the best model but was manually added to examine its effect and formally test the second additional hypothesis.

Analysis revealed a significant effect of *Scenario* on participants' pain beliefs. Specifically, participants exposed to Scenario 2 were significantly less likely to believe in paralarval pain, compared to those exposed to Scenario 3 ($p = 0.0017$; $**p \leq 0.01$). Surprisingly, there was no significant difference observed between Scenario 1 and Scenario 2 ($p = 0.11$), nor between Scenario 1 and Scenario 3 ($p = 0.36$). These results, therefore, do not support the first additional hypothesis, as participants exposed to Scenario 1 were not significantly more likely to believe paralarvae are capable of experiencing pain, compared to participants exposed to Scenario 2 and participants exposed to Scenario 3. These results are visualized in Figure 12 (next page).

Additionally, the analysis revealed there was no significant effect of *Group* on participants' pain beliefs ($p = 0.37$). The proportion of participants who believed that paralarvae can experience pain was nearly identical across all three participant groups, revealing no significant difference between cephalopod biologists and other biologists ($p = 0.52$), nor between cephalopod biologists and non-biologists ($p = 1$), nor between other biologists and non-biologists ($p = 1$). These results are visualized in Figure 13. Similarly, familiarity with research ethics (*RE_High*) also did not have a significant effect on the proportion of participants believing *O. vulgaris* paralarvae are capable of experiencing pain.

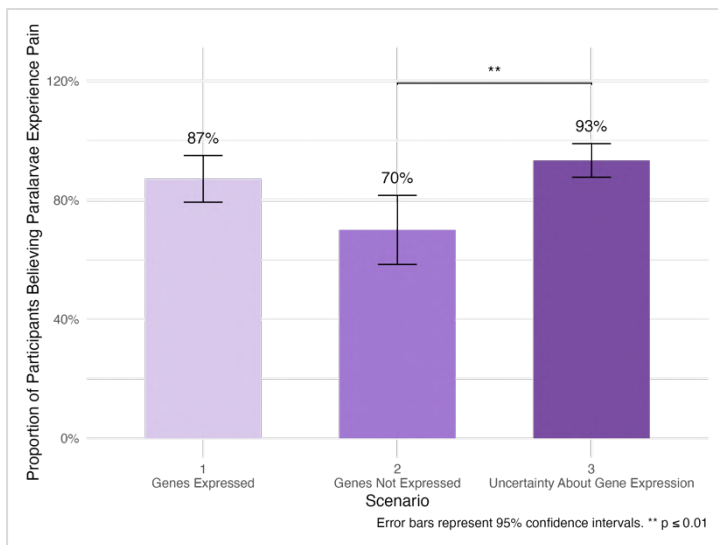


Figure 12: Effect of the received scientific background information (Scenario) on the proportion of participants believing that *O. vulgaris* paralarvae are capable of experiencing pain. The proportion of participants indicating belief in paralarval pain experience was 87% for participants exposed to Scenario 1 (genes expressed), 70% for participants exposed to Scenario 2 (genes not expressed), and 93% for participants exposed to Scenario 3 (uncertainty about gene expression). Participants exposed to Scenario 2 were significantly less likely to believe paralarvae are capable of experiencing pain, compared to those exposed to Scenario 3 (** $p \leq 0.01$). There were no significant differences observed between Scenario 1 and the other two Scenarios. Error bars represent 95% confidence intervals.

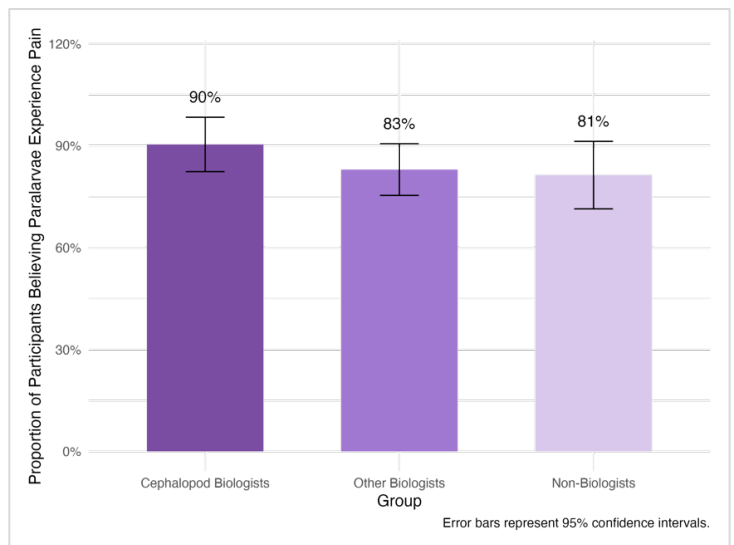


Figure 13: Effect of the participant group on the proportion of participants believing that *O. vulgaris* paralarvae are capable of experiencing pain. The proportion of participants believing paralarvae are capable of experiencing pain was 90% for cephalopod biologists, 83% for other biologists, and 81% for non-biologists. There were no significant differences observed between the groups. Error bars represent 95% confidence intervals.

5. Discussion

5.1. Understanding What Influenced Ethical Judgment

It was expected that participants exposed to Scenario 1, in which nociception-related genes were expressed, would be more likely to rate the research as “unethical.” This was based on the assumption that the expression of these genes would lead participants to infer that *O. vulgaris* paralarvae are capable of experiencing pain. However, the results did not support this hypothesis, as the provided scientific context had no overall effect on participants’ ethical judgments. A possible explanation is that participants either did not infer pain capacity from the gene expression data or did not view this as sufficient evidence for potential pain experience in *O. vulgaris* paralarvae.

Furthermore, it was hypothesized that participants who believed further research is needed to resolve uncertainty about pain in *O. vulgaris* paralarvae would be more likely to rate the research as “unethical,” compared to those who did not hold this belief. However, this was not supported by the results. A possible explanation lies in a bias introduced by the survey design. At the beginning of the survey, participants read an abstract describing a gene expression experiment investigating nociception—the process by which potentially painful stimuli are detected and processed—in *O. vulgaris* paralarvae. After, they were asked a series of questions, including how ethical they considered this type of research and whether more research about pain perception in *O. vulgaris* paralarvae is needed. Since the abstract already described research aiming to resolve uncertainty about pain, participants may have conceptually linked these two questions, responding that further research is needed when they viewed the study as ethical, and that no more research is needed when they did not. This interpretation is supported by the finding that participants who believed no further research was needed were also slightly more likely to rate the research as unethical. Future studies should consider more clearly separating introductory materials from follow-up questions to reduce potential bias and better isolate participants’ reasoning.

Surprisingly, familiarity with research ethics also did not significantly affect participants’ ethical ratings. It was expected that those reporting higher levels of familiarity would be less likely to rate the research as “unethical,” as they might be aware that *O. vulgaris* paralarvae are already protected under legislation and that the described experiment complies with existing ethical standards. However, this expectation was not supported by the results, raising important questions about how ethics training translates into actual ethical reasoning. One possible explanation for this observation is that the study relied on self-reported familiarity with research ethics, instead of formally assessed familiarity. Since participants assessed their own level of familiarity, it is plausible that some overestimated their knowledge. To better evaluate the impact of ethics training on ethical judgment, future studies should incorporate more objective measures of ethical knowledge and formal instruction.

Notably, the results did reveal a significant difference between non-biologists, other biologists, and cephalopod biologists in their ethical ratings of the research. Non-biologists were most likely to rate the research as “unethical,” challenging previous claims that invertebrates are typically neglected by the public due to being perceived as inferior, simplistic, or “alien” (Horvath et al., 2013; Lockwood, 2014; Mather, 2019; Howard & Symonds, 2020; Iglesias, 2021). However, it is important to consider that octopuses may not be representative of invertebrates as a whole. While invertebrates are often overlooked due to their lack of emotional expression, cognitive complexity, or sophisticated nervous systems (Iglesias, 2021), cephalopods, including *O. vulgaris*, have increasingly stood out as a notable exception. Extensive research has highlighted their advanced cognitive abilities, complex behavioral repertoires, and highly developed nervous systems (Mather & Dickel, 2017; Schnell et al., 2021; Ponte et al., 2021; Albertin & Katz, 2023; Jozet-Alves et al., 2023). This growing scientific attention has not only elevated their status in neurobiological and behavioral research but may also have shaped public perception, potentially prompting participants to regard octopuses more similarly to vertebrates (Crook, 2021; Dominguez-Lopez et al., 2021; Almeida et al., 2022). Additionally, although unlikely, it is possible that some individuals were not fully aware that octopuses are, in fact, invertebrates, which may have influenced their responses.

In contrast to non-biologists, cephalopod biologists were significantly less likely to rate the research as “unethical,” and ethical ratings from other biologists fell in between, without differing significantly from either group. While one might expect that greater familiarity with research ethics could explain the variation between groups, this factor did not significantly influence ethical ratings, as explained above. This suggests that the group differences observed here are more likely rooted in domain-specific expertise and direct experience with the animal, rather than general knowledge of ethical guidelines. Cephalopod biologists, who were least likely to rate the research as “unethical,” are also the participants most familiar with *O. vulgaris* paralarvae and with the fact that these animals are already protected under legislation. As such, their background knowledge, hands-on experience with the organism (or closely related species), and trust in the regulatory framework may have contributed to their perception that the research was ethically justified. Slightly more biologists working with other organisms rated the research “unethical,” though not significantly more. This may reflect a similar trust in ethical standards, tempered by less direct familiarity with *O. vulgaris*. Their limited experience with cephalopods could have introduced some uncertainty about whether the procedures described were appropriate, resulting in a modest increase in ethical concern. In contrast, non-biologists, who lack a scientific background and direct experience with the subject organism, may have relied primarily on moral intuition and empathy-based reasoning. For them, labeling the research as “unethical” may have represented the safer or more morally conservative choice, especially in the absence of full understanding.

Overall, the more familiar participants were with the animal and research context, the less likely they were to rate the research as “unethical.” This suggests that domain-specific knowledge shaped participants’ ethical judgments more strongly than the provided scientific context or self-rated familiarity with research ethics, neither of which had a significant effect on ethical ratings. These findings raise important questions about how scientific information is communicated and interpreted by the public, as well as about the effectiveness of current ethics training.

5.2. Understanding What Influenced Agreement With Societal Relevance Outweighing Pain

As expected, participants who believed that *O. vulgaris* paralarvae are capable of experiencing pain were significantly less likely to agree that the societal relevance of the research outweighs this pain, compared to those who did not hold this belief. This may be because participants who believe in paralarval pain experience expressed greater compassion toward the animals and were less willing to justify this pain based on perceived societal benefits. In contrast, those who did not believe paralarvae can experience pain likely did not perceive the situation as a genuine ethical conflict. For them, only the societal relevance of the research remained salient, making them more inclined to prioritize it.

An additional significant effect, not originally hypothesized, was that of gender. Male participants were significantly more likely to agree that societal relevance outweighs the potential pain experienced by *O. vulgaris* paralarvae, compared to females. This may reflect a broader tendency among men to approach ethical decisions with less emotional sensitivity or caution. Prior research supports this pattern as women tend to behave more ethically across a range of contexts (Shonk, 2025). Women also score higher on empathy, regardless of cultural background, and are more risk-averse than men (Block, 2023; Psychological Consultancy Ltd., 2016). These traits may make female participants more attuned to the ethical implications of potential animal pain and more reluctant to justify it solely based on societal benefit.

Once again, neither familiarity with research ethics nor the scientific context provided had a significant effect. It was expected that participants who were highly familiar with research ethics would be more likely to accept that the societal relevance of the research outweighs the potential pain experienced by *O. vulgaris* paralarvae, compared to participants with low familiarity. However, no such difference was observed. As mentioned earlier, this may be explained by the use of self-rated familiarity with research ethics, which could have resulted in an overestimation of their knowledge. To determine whether this is indeed the case or whether familiarity with research ethics genuinely does not influence ethical perceptions and reasoning, future research should include a formal assessment of participants’ knowledge of research ethics.

In addition, it was expected that participants exposed to Scenario 1, in which nociception-related gene expression was observed, would be less likely to agree that societal relevance outweighs potential pain. However, this was not supported by the results. Similarly, as discussed earlier, participants may not have interpreted the presence of these genes as evidence of possible pain experience in *O. vulgaris* paralarvae. This interpretation is supported by the fact that participants who believed paralarvae are capable of experiencing pain were less likely to agree that the societal relevance outweighs this pain. If participants had associated the gene expression data with the possibility that the paralarvae might be capable of experiencing pain, a comparable pattern would likely have emerged when participants were exposed to Scenario 1.

5.3. Understanding What Influenced Belief in the Need for Further Research

It was anticipated that participants who viewed the research as highly valuable to society would be less likely to support the need for further investigation of pain perception in *O. vulgaris* paralarvae. However, this expectation was not supported. Regardless of how societally relevant participants perceived the research to be, the majority still supported further investigation, with 79% of those who rated societal relevance as “low” and 81% of those who rated it as “high” indicating that more research is needed. This suggests that perceived societal relevance does not substantially influence whether participants believe additional research is necessary, which is unexpected, as it suggests that participants are bad at trade-off thinking.

Interestingly, a significant effect of gender was also observed in this context. Although both male (72%) and female (87%) participants highly supported the need for further research, female participants were significantly more likely to say that further research is necessary to resolve uncertainty about pain experience in *O. vulgaris* paralarvae. As previously discussed, this may reflect broader gender-based differences in ethical decision-making, where women tend to show greater empathy and risk awareness in morally uncertain situations (Shonk, 2025; Block,

2023; Psychological Consultancy Ltd., 2016).

5.4. Understanding What Influenced Confidence in Pain Interpretation

It was anticipated that the level of confidence would increase alongside the academic experience, as higher academic experience would enable participants to interpret complex scientific information with more ease and certainty. In contrast, those without such a background might feel less equipped to assess these issues, resulting in lower confidence. The results partially supported this hypothesis. Participants with high academic experience were significantly more likely to report high confidence compared to those with low experience. This suggests that extensive scientific training may increase confidence, likely due to greater familiarity with interpreting complex information. Participants with less experience may be more cautious, avoiding overstatement when judging uncertain evidence.

However, no significant difference was found between participants with high academic experience and those with no such experience (i.e., non-biologists). Among non-biologists, 46% reported high confidence in their interpretation of paralarval pain experience, compared to 53% among those with high academic experience. Although non-biologists were slightly less likely to report high confidence, the difference was not significant. Additionally, there was also no significant difference observed between non-biologists and participants with low academic experience. This pattern suggests that non-biologists are more confident than expected when compared to biologists with fewer years of academic experience, but not quite reaching the confidence levels of the most experienced participants. One interpretation is that, lacking scientific training, non-biologists may overestimate their ability to evaluate complex biological evidence. They may be unaware of the depth of knowledge required to confidently assess pain-related gene expression, leading to a level of confidence that appears inflated relative to their background. This overconfidence aligns with the Dunning-Kruger effect, whereby individuals with limited expertise tend to overestimate their competence (Kruger & Dunning, 1999). At the same time, the observed pattern suggests that confidence does not increase linearly with experience.

5.5. Understanding What Influenced Belief in Paralarval Pain

Against expectations, participants exposed to Scenario 1, in which nociception-related gene expression is observed, were not significantly more likely to believe that paralarvae are capable of experiencing pain, compared to participants exposed to the scenario in which this gene expression is not observed and participants exposed to unresolved uncertainty. This supports the notion that participants may not have inferred the possibility of pain from the presence of nociception-related genes, suggesting difficulty in understanding the implications of the experiment and highlighting challenges in interpreting complex scientific findings.

More unexpectedly, participants presented with the scenario describing unresolved uncertainty, which best reflects the current state of scientific understanding regarding paralarval pain, were the most likely to believe that paralarvae are capable of experiencing pain. When exposed to this scenario, 93% of participants indicated a belief in paralarval pain, compared to 87% in the condition where nociception-related gene expression was observed. While this difference was not statistically significant, it is still notable. When faced with uncertainty, these participants may have adopted a more cautious stance, granting paralarvae the benefit of the doubt. This response aligns with the precautionary principle, which states that when evidence for sentience is inconclusive, animals should nonetheless be treated as if they are capable of experiencing pain (Birch, 2017).

A significant difference did emerge, however, between the uncertainty condition and the scenario in which no nociception-related gene expression was observed. Only 70% of participants in the latter condition believed that paralarvae can experience pain. Although still a majority, this considerably lower proportion may reflect a tendency to interpret the absence of gene expression as indirect evidence for the absence of nociception, and by extension, the capacity to experience pain. Yet, this conclusion is scientifically premature, as the absence of confirmed gene expression does not preclude the existence of other nociceptive mechanisms. Given the complexity of such molecular evidence, this nuance may not have been readily apparent to many participants.

Finally, it was also hypothesized that cephalopod biologists would be more likely to believe that paralarvae are capable of experiencing pain, compared to other participant groups. While belief levels followed the expected pattern—highest among cephalopod biologists, followed by other biologists, and lowest among non-biologists—these differences were not significant. The fact that non-biologists were just as likely as both biologist groups to believe that *O. vulgaris* paralarvae can experience pain once again challenges the assumption that invertebrates are viewed as inferior among the general public (Horvath et al., 2013; Lockwood, 2014; Mather, 2019; Howard & Symonds, 2020; Iglesias, 2021). However, as explained before, it is important to remember that octopuses, such as *O. vulgaris*, may not be representative of invertebrates as a whole.

5.6. Limitations of the Study

While the findings of this study offer valuable perspectives, they should be interpreted in light of several limitations. Both familiarity with research ethics and English language proficiency were self-reported, which may have introduced bias. Participants might have overestimated their level of understanding or language skills, leading to potential misclassification in the analysis. For instance, participants who reported high familiarity with research ethics may in fact have had limited knowledge, resulting in their inclusion in the “high familiarity” group despite lacking the corresponding expertise. This could have affected the observed relationships between ethical familiarity and participants’ responses. Similarly, a cut-off score of 3 out of 5 was used to exclude participants with insufficient English proficiency, based on the assumption that those below this threshold might not have fully understood the survey questions. However, if some participants overestimated their proficiency, it remains possible that miscomprehension still occurred, even within the included sample. To avoid such issues, future research should formally assess both research ethics familiarity and English language proficiency using objective measures.

Another limitation relates to the use of a snowball sampling strategy, which prevented the calculation of an exact response rate and limits the generalizability of the findings. As is common in this type of survey-based research, the representativeness of the sample remains uncertain. Additionally, participant engagement with the experimental material was not monitored. Specifically, the time participants spent reading the abstracts was not recorded, raising the possibility that some may not have fully absorbed the information presented. This may help explain why the scientific context did not significantly influence ethical judgments. Future studies should consider tracking reading time or incorporating attention checks to ensure sufficient exposure to the experimental conditions.

A further limitation lies in a potential design-related bias introduced by the sequence of materials. At the beginning of the survey, participants read an abstract describing a gene expression experiment investigating nociception in *O. vulgaris* paralarvae. Afterward, they were asked a series of questions, including how ethical they considered this type of research and whether more research about pain perception in *O. vulgaris* paralarvae is needed. Since the abstract already described research aiming to resolve uncertainty about pain, participants may have conceptually linked these two questions, responding that further research is needed when they viewed the study as ethical, and that no more research is needed when they did not. Future studies should consider more clearly separating introductory materials from follow-up questions to reduce potential bias and better isolate participants' reasoning.

It is also worth noting that the responses concerning *O. vulgaris* paralarvae may not be representative of public attitudes toward invertebrates more broadly. In recent years, there has been a noticeable increase in scientific attention and interest in cephalopods, particularly octopuses, due to their advanced cognitive abilities, behavioral complexity, and expanding use as model organisms in neuroscience and developmental biology. This growing awareness may have contributed to participants attributing higher moral consideration to these animals compared to other invertebrate species.

Finally, the finding that participants did not infer pain capacity from gene expression points to a deeper issue in how scientific findings are communicated and interpreted. Future research should examine how different formats (e.g., visual aids, simplified explanations, analogies) affect public understanding and ethical reasoning, particularly in contexts involving scientific uncertainty. Longitudinal or qualitative approaches could also provide richer insight into how ethical views evolve as new evidence emerges or is presented in more accessible ways.

6. Conclusions and Future Perspectives

This study revealed that scientific evidence, specifically the presence of nociception-related gene expression, did not significantly influence participants' ethical ratings, belief in paralarval pain, or acceptance of the ethical trade-off between societal relevance and the potential pain experienced by paralarvae. This suggests that such information was not clearly understood or not regarded as meaningful when forming ethical judgments. Instead, belief in the pain capacity of paralarvae emerged as a much stronger predictor. Participants who believed that *O. vulgaris* paralarvae can experience pain were significantly more likely to consider the research unethical, and less likely to agree that its societal relevance outweighs this pain. Furthermore, cephalopod biologists were the least likely to rate the research as “unethical,” possibly due to their background knowledge, hands-on experience with cephalopods, and trust in the regulatory framework. Additionally, a significant effect of gender was observed, with female participants being significantly less likely to prioritize societal relevance over potential pain, and more likely to support further investigation to resolve uncertainty about pain in paralarvae. Self-reported familiarity with research ethics, on the other hand, did not significantly affect responses, raising questions about how ethics training translates into actual ethical reasoning. Also, the proportion of participants reporting high confidence in their interpretation of paralarval pain was highest in the group with extensive academic experience. However, this proportion was not significantly higher compared to the one reported by participants with no academic experience (i.e., non-biologists). This may reflect an overestimation of non-biologists' interpretive ability, consistent with the Dunning–Kruger effect, whereby individuals with limited expertise tend to overestimate their competence. Finally, despite common assumptions that invertebrates are broadly dismissed as inferior, non-biologists in this study were just as likely as biologists to believe in paralarval pain and were the most ethically cautious overall.

Together, these findings suggest that ethical judgments in complex scientific contexts are shaped more by intuitive beliefs, disciplinary experience, and gender than by scientific evidence and familiarity with research ethics. This underscores the need for more effective science communication and ethical engagement, particularly when evidence is uncertain, to support better-informed, inclusive decision-making in animal research governance. Future studies should explore how the format and framing of scientific evidence, such as the use of visualizations, simplified summaries, or analogies, can enhance public comprehension and ethical reasoning. Additionally, more robust approaches to assessing participants' background knowledge, language proficiency, and engagement with experimental material are needed to ensure data quality and interpretability. Investigating how participants process complex ethical trade-offs over time, or in real-world policy contexts, may also help clarify how public views evolve in response to emerging scientific information.

Expression Patterns of Nociception-Related Genes in the Paralarval *Octopus vulgaris*

Microthesis Biology

3B. Materials and Methods

In this experiment, the expression patterns of several genes potentially involved in nociception were examined in *Octopus vulgaris* paralarvae. To observe possible developmental effects in the expression patterns of these genes, expression was analyzed at two developmental stages of the paralarvae, including 5 days post-hatching (dph) and 40 dph. 5 dph represents the stadium short after hatching, whereas 40 dph typically corresponds to one of the latest stages achievable under laboratory conditions, given the known difficulties in long-term paralarval rearing (Iglesias et al. 2007; Birch et al., 2021; M. Lanoizelet, personal communication, 2024). For this study, *O. vulgaris* paralarvae were raised and maintained under controlled laboratory conditions. Paralarvae were kept in recirculating systems containing artificial seawater (Tropic Marin® Sea Salt Classic) with stable water parameters: salinity of 35g/L, pH 8.0, and temperature maintained between 19.5°C and 20.5°C. A 14:10h light:dark photoperiod was applied, and live *Artemia* was provided twice a day, every day at 09:00 and 18:00. Tank maintenance included daily cleaning via gentle siphoning to remove waste and uneaten food. Handling of individuals was performed using fine plastic or glass Pasteur pipettes to minimize mechanical stress. For experimental procedures, paralarvae were first sedated in a 2% ethanol solution prepared in artificial seawater, and subsequently anesthetized in a 4% ethanol solution prior to fixation or further processing.

Several genes were selected for inclusion in the experiment based on their known or proposed roles in nociceptive pathways. The selection of these genes was informed by two main sources: (1) a study on adult *Octopus vulgaris* that aimed to map putative nociceptors in the octopus arm (Di Cristina, 2017), and (2) available gene expression data from the 1 dph *Octopus vulgaris* single-nucleus RNA sequencing (snRNAseq) dataset provided by the Seuntjens Lab (KU Leuven). The combination of these two sources, along with practical considerations related to probe design and gene expression, led to the final selection of six genes: *Kcnk1*, *Trpa1*, *Asic1*, *Ano1/2*, *Oprm2*, and *Piezo2*. The gene *Ano1/2* represents a merged designation due to limited annotation resolution, which prevented a clear distinction between *Ano1* and *Ano2* transcripts at the probe design stage. More information about these genes, their function, and related stress role, can be found in Table 1 (next page).

Table 1: Overview of the candidate genes selected for investigating potential involvement in nociception in *Octopus vulgaris* paralarvae. This table summarizes each gene's full name, molecular classification, structural characteristics, biological function, and (presumed) stress-related role. Gene selection was guided by findings from a study on putative nociceptors in adult *O. vulgaris* (Di Cristina, 2017) and their expression in a single-nucleus RNA sequencing (snRNAseq) dataset of 1 dph *O. vulgaris* provided by the Seuntjens Lab (KU Leuven). References indicate key sources for further information on each gene and its function. Full citations are provided in Section 7 (References, pages 52 – 66)

Gene	Full Name	Molecule Type	Structure	Function	Stress Role	References
<i>Kcnk1</i>	Potassium channel subfamily K member 1	Ion channel protein	Two-pore domain potassium (K2P) channel, tetrameric	Allows potassium ions (K ⁺) to leak across the cell membrane, stabilizing resting membrane potential and reducing excitability	Modulated by oxidative, heat, and mechanical stress, which can alter its conductance	Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015
<i>Trpa1</i>	Transient receptor potential ankyrin 1	Non-selective cation channel	TRP (transient receptor potential) channel family member with ankyrin repeats	Permits influx of Ca ²⁺ and Na ⁺ ions in response to noxious stimuli like oxidants, heat, and electrophilic compounds	Major nociceptive sensor, involved in inflammatory pain, oxidative stress sensing, and possibly thermosensation	Nagata et al., 2005; Tai et al., 2008; Laursen et al., 2015; Manolache et al., 2021
<i>Ano1/2</i>	Anoctamin 1 and 2	Calcium-activated chloride channel	Transmembrane protein with 8-10 membrane-spanning domains	Facilitates Cl ⁻ ion flow across membranes in response to intracellular Ca ²⁺ rise	Contributes to neuronal excitability and secretory activity; modulated by inflammatory signals and may act as a thermal sensor in nociceptive pathways	Cho et al., 2012; Cho & Oh, 2013; Lee et al., 2015; Auer et al., 2021; DiBattista et al., 2024
<i>Asic1</i>	Acid-sensing ion channel 1	Voltage-insensitive, ligand-gated ion channel	Member of the DEG/ENaC (family; forms homo- or heterotrimeric channels	Activated by extracellular protons (H ⁺) under acidic conditions (e.g., tissue damage), allowing Na ⁺ influx	Detects acidification and contributes to the perception of pain, especially chemical and thermal nociception	Paukert et al., 2004; Gu & Lee, 2010
<i>Oprm2</i>	Mu-type opioid receptor 2	G protein-coupled receptor (GPCR)	7-transmembrane domain receptor	Activated by endogenous opioids (e.g., endorphins) and exogenous compounds (e.g., morphine); inhibits cAMP production and modulates ion channel activity	Mediates analgesia by reducing nociceptive transmission; also involved in stress regulation, emotional processing, and reinforcement pathways	Ossipov et al., 2010; Dhalliwal & Gupta, 2023; Cao et al., 2024
<i>Piezo2</i>	Mechanosensitive ion channel component 2	Mechanically-gated ion channel	Large trimeric transmembrane protein with a unique curved, propeller-like structure	Activated by mechanical forces such as stretch and pressure; permits influx of cations (mainly Ca ²⁺ and Na ⁺)	Essential for innocuous touch sensation; contributes to mechanical nociception and proprioception; recent evidence shows relief of voltage block can sensitize nociceptors in chronic pain contexts	Kim et al., 2012; Florez-Paz et al., 2016; Anderson et al., 2017; Sánchez-Carranza et al., 2024

Gene expression patterns were visualized in whole mount *Octopus vulgaris* paralarvae using hybridization chain reaction (HCR) v3.0, a highly sensitive *in situ* hybridization technique that enables multiplexed detection of RNA transcripts in fixed tissue sections (Choi et al., 2018; Schwarzkopf et al., 2021). This method relies on the use of DNA probes specific to the target mRNA sequences, which initiate a chain reaction of self-assembling fluorescent DNA hairpins, resulting in signal amplification without the use of enzymes (Choi et al., 2018; Schwarzkopf et al., 2021). A more detailed explanation of this process can be found in Figure 14. Probe pairs for HCR v3.0 were designed using the automated tool *Easy_HCR* (Seuntjens, 2021).

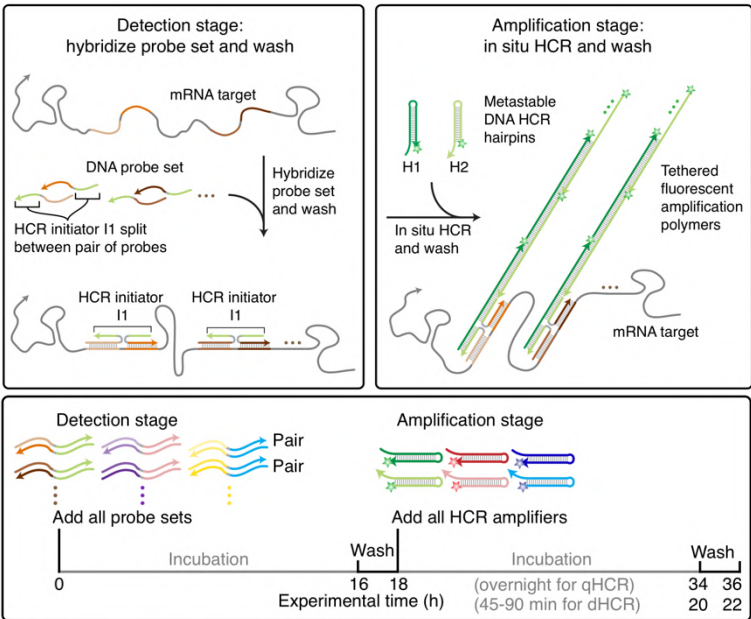


Figure 14: Overview of the Hybridization Chain Reaction (HCR) v3.0 protocol for *in situ* mRNA detection (Choi et al., 2018). The HCR v3.0 workflow consists of two main stages: a detection stage and an amplification stage. During the detection stage (top left), split DNA probe pairs hybridize to specific regions of the target mRNA, positioning the HCR initiator (I1) in place. After hybridization and washing, the amplification stage (top right) is initiated by the addition of metastable fluorescent DNA hairpins (H1 and H2). These hairpins undergo a chain reaction triggered by the initiator sequence, forming tethered fluorescent amplification polymers at the target site. The timeline (bottom) illustrates the incubation and washing steps typical for quantitative HCR (qHCR) and digital HCR (dHCR) protocols, including multiplexed probe and amplifier addition. This method enables sensitive, enzyme-free, and multiplexed visualization of gene expression in fixed tissues.

For performing HCR, the protocol described by Elagoz et al. (2022) was followed with some minor adjustments. Paralarval *Octopus vulgaris* specimens were fixed overnight at 4°C in 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS) treated with diethylpyrocarbonate (DEPC) to eliminate RNase activity. After, samples were washed thoroughly and stored in PBS at 4°C until further processing. Tissues were subsequently embedded in paraffin (Figure 15A) and sectioned at a thickness of 6 µm using a microtome (Figure 15B, Figure 15C). Sections were mounted onto Superfrost Plus™ microscope slides, ensuring that as many sections as possible were placed on each slide (Figure 15D). The sectioning plane was checked prior to HCR to confirm orientation and quality.

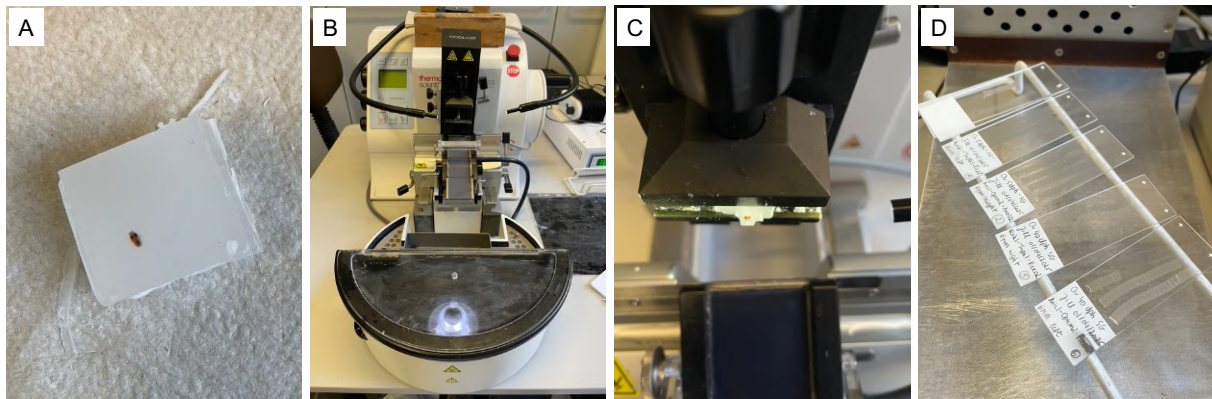


Figure 15: Sectioning workflow of *Octopus vulgaris* paralarvae for HCR. (A) *Octopus vulgaris* paralarva embedded in paraffin, prior to sectioning. (B) Microtome setup used to cut tissue sections at a thickness of 6 µm. (C) Trimming of excess paraffin around the paralarva to minimize space usage and maximize the number of sections per slide. (D) *Octopus vulgaris* paralarvae sections mounted onto Superfrost Plus™ microscope slides; the left and right halves of each paralarva were placed on separate slides to maintain consistent orientation and distribution.

Prior to the start of the HCR experiment, all glassware was washed in a laboratory dishwasher and baked overnight at 180°C to prevent RNase contamination (note: plastic items were not baked). All tools and surfaces, including tweezers, humidified chambers, plastic lids, and the working bench were decontaminated using RNase AWAY™, incubated for 5–10 minutes, and wiped clean with RNase-free tissue paper. RNase-free reagents and plasticware were used throughout the experiment. All work was carried out in RNase-free conditions to prevent degradation of mRNA. For safety, all steps involving formamide were performed under a chemical fume hood. Glassware used in these steps was left to dry under the hood before further handling.

On the first day of the HCR experiment, deparaffinization, rehydration, permeabilization, and hybridization steps were performed. To begin the experiment, slides were baked in a hybridization oven at 65°C for 30 minutes. Subsequently, slides were immersed in various solutions for deparaffinization using a glass container. These steps were performed under an RNase-free hood. First, slides were immersed for 4 minutes in 100% xylene, followed by another 4 minutes in fresh 100% xylene.

After gentle blotting to remove excess xylene, slides were transferred to 100% ethanol for rehydration, with three consecutive 4-minute immersions. Slides were carefully dried between transitions to ensure optimal solvent exchange. After, slides were baked again at 65°C for 5 minutes, after which the oven temperature was reduced to 37 °C in preparation for subsequent steps. Meanwhile, a humidified chamber was prepared using autoclaved Milli-Q water. After baking, a hydrophobic barrier was drawn around the tissue sections on the slide using a PAP pen to prevent buffer spreading, after which the slides were air-dried for 5 minutes. Permeabilization was carried out by incubating slides with 1 mL of proteinase K solution—prepared by diluting 1 µL of proteinase K in 3 mL of PBS-DEPC—per slide in the humidified chamber at 37°C for 5 minutes. This temperature was maintained to facilitate enzymatic activity. Slides were then washed twice for 2 minutes each with RNase-free water, ensuring full coverage of the sections during each wash.

For hybridization, both the hybridization oven and the probe hybridization buffer were pre-warmed to 37°C. The residual buffer was carefully removed from each slide. After, 200 µL of pre-warmed probe hybridization buffer—containing 30% formamide, 5x SSC, 9 mM citric acid pH 6, 0.1% Tween 20, 50 µg/mL heparin, 1x Denhardt's solution, and 10% dextran sulfate—was added to each slide under a chemical fume hood, and a piece of parafilm was placed on top to evenly distribute the solution. Slides were pre-hybridized for 30 minutes at 37°C in a humidified chamber. During this incubation, the probe mixtures could be prepared. Custom-designed DNA probe sets targeting the selected genes—*Kcnk1*, *Trpa1*, *Asic1*, *Ano1/2*, *Oprm2*, and *Piezo2*—were used to initiate the hybridization chain reaction upon binding to their complementary mRNA targets. Each gene was assigned a distinct amplifier hairpin set (B1, B2, or B3), each conjugated to a specific fluorophore (B1 = yellow, B2 = pink, and B3 = blue), allowing for the simultaneous detection of up to three genes per sample. Probe mixtures were thawed on ice and centrifuged. Since we were working with new probes, the probe solution was prepared by adding 1.8 pmol of each probe mixture to 75 µL of pre-warmed probe hybridization buffer per slide. After removing the pre-hybridization solution, 75 µL of the probe solution was added to each slide, followed by the application of a coverslip (Hybrislip®) to ensure even distribution. Slides were incubated overnight (12-16 hours) at 37°C in a humidified chamber.

The following day of the HCR experiment, wash and amplification steps were performed. To begin, coverslips were carefully removed by holding the slides vertically, allowing the coverslip to slide off, ensuring minimal disturbance to the tissue. If needed, slides could be immersed in probe wash buffer—containing 30% formamide, 5x SSC, 9 mM citric acid pH 6, 0.1% Tween 20, and 50 µg/mg heparin—to allow the coverslip to lift off. As the buffer contains formamide, this step was carried out under a chemical fume hood. Fresh Whatman paper was placed in the humidified chamber in preparation for subsequent incubations.

To remove excess probes, slides were incubated at 37°C in a glass Coplin jar through a series of buffer exchanges. First, slides were immersed for 15 minutes in a solution containing 25% 5x SSCT and 75% probe wash buffer. This was followed by a 15-minute incubation in a 50:50 mixture of 5x SSCT and probe wash buffer. Next, slides were incubated for 15 minutes in a solution consisting of 75% 5x SSCT and 25% probe wash buffer. Finally, slides were incubated for 15 minutes in 100% 5x SSCT to complete the wash series. This was followed by another final wash in 100% 5x SSCT for 5 minutes at room temperature. During this process, hairpins were thawed on ice in the dark, and the amplification buffer—containing 5x SSC, 0.1% Tween 20, and 10% Dextran sulfate—was brought to room temperature.

For the amplification step, residual SSCT buffer was removed, and hydrophobic barriers were reinforced with a PAP pen if necessary. A total of 500 µL of amplification buffer was added to each slide, followed by pre-amplification in a dark, humidified chamber at room temperature for a minimum of 30 minutes. Hairpin preparation was performed in parallel. For each slide, 9 pmol of hairpins H1 and H2 were prepared by snap-cooling 1.5 µL of 3 µM stock solution of each hairpin (equivalent to 4.5 pmol) in separate PCR tubes. Hairpins were kept separately to prevent premature hybridization. Snap-cooling was performed by heating the tubes at 95°C for 90 seconds in a thermocycler. Immediately after heating, the tubes were transferred to ice and incubated for 5 minutes in the dark. Following this step, the hairpins were left to equilibrate at room temperature for 30 minutes to allow proper folding into their active secondary structures. This step was carried out in the dark to protect the fluorophores from light degradation.

After the snap-cooling procedure, the hairpin amplification solution was prepared. For each slide, a total volume of 75 µL hairpin amplification solution—consisting of both hairpins and amplification buffer—was required. Per slide, 1.5 µL of each hairpin (H1 and H2) was used for each of the three fluorescent channels (B1, B2, and B3), resulting in a total volume of 9 µL hairpin solution per slide. To reach the required total volume of 75 µL hairpin amplification buffer per slide, an additional 66 µL of room-temperature amplification buffer was added. The combined hairpin solution was gently mixed and kept at room temperature until application. After, 75 µL of the freshly prepared hairpin solution was applied to the tissue sections. Finally, a Hybrislip® was placed on top to ensure even distribution, and slides were incubated overnight (12-16 hours) in a dark, humidified chamber at room temperature.

On the third and final day of the HCR experiment, mounting of the slides was performed. To begin, coverslips (Hybrislips®) were gently removed by holding the slides vertically, allowing them to slide off under gravity. As the tissue sections were 6µm thick, this did not cause any damage. If the coverslip did not detach easily, slides were immersed in 5x SSCT at room temperature to facilitate its removal by floating the coverslip off.

To remove excess hairpins, slides were incubated three times for 10 minutes each in fresh 5x SSCT at room temperature. No additional steps, such as immunohistochemistry (IHC), were performed following this HCR protocol.

For mounting, 500 μ L of SSCT-DAPI (1:1000 dilution) was applied to each slide, followed by a 20-minute incubation in the dark. Slides were then washed for 5 minutes in 5x SSCT to remove unbound DAPI. Subsequently, 70 μ L of Mowiol mounting medium was added directly onto the tissue sections. A glass coverslip was carefully placed on top, while preventing air bubbles from forming underneath, as this could otherwise lead to drying and damage of the tissue sections. Due to the adhesive nature of Mowiol and the possibility of leakage during storage, aluminum foil was placed inside the slide container. This prevented the slides from sticking to the container, which could otherwise result in damage and render them unsuitable for imaging. Prepared slides were allowed to dry in a temperature-controlled wine refrigerator and were subsequently stored at 4°C, protected from light.

Fluorescent signals were visualized using the ZEISS LSM 900 laser scanning confocal microscope (LSCM). Images were acquired with a 20x magnification and standardized settings. Laser intensities were set per fluorescence channel at the beginning of each imaging session and kept constant across all developmental stages and probes to ensure comparability of raw image data. Three fluorescent channels were used: B1, excited at 546 nm and visualized in yellow; B2, excited at 647 nm and visualized in pink; and B3, excited at 488 nm and visualized in blue. Post-acquisition processing (e.g., brightness and contrast adjustments) was performed using Fiji v2.16.0 (Schindelin et al., 2012), based on ImageJ 1.54p. To enhance clarity and allow for visual comparison, images were adjusted individually using Fiji's Min/Max scaling and manual brightness/contrast tuning to ensure clear visibility of the signal. While post-processing parameters were not identical across all images, adjustments were made following common practices to optimize signal visibility.

4B. Results

As a reference point for anatomical context, an overview of key morphological structures at 40 dph is provided in Figure 16 and Figure 17. While all labeled structures are also present at 5 dph, the 40 dph stage was selected for this overview due to the improved clarity and visibility of anatomical features at this later developmental stage.

Figure 16 shows the expression patterns of *Asic1*, *Oprm2*, and *Piezo2*, detected using hybridization chain reaction (HCR) and visualized by laser scanning confocal microscopy. DAPI was used as a nuclear counterstain. This figure not only highlights several key anatomical structures but also draws attention to one of the major challenges of HCR imaging: autofluorescence. Certain organs, such as the Kölliker organs, the hepatopancreas, the beak, and the radula (not visible in Figure 16), are typically autofluorescent. As a result, it remains uncertain whether true gene expression signals are present in these areas. Distinguishing genuine HCR signals from autofluorescence is essential. True HCR signals usually appear as distinct, punctuate "dots," while auto-fluorescence tends to produce more diffuse or smeared fluorescence. Careful interpretation of these patterns is necessary to avoid misidentifying autofluorescence as gene expression.

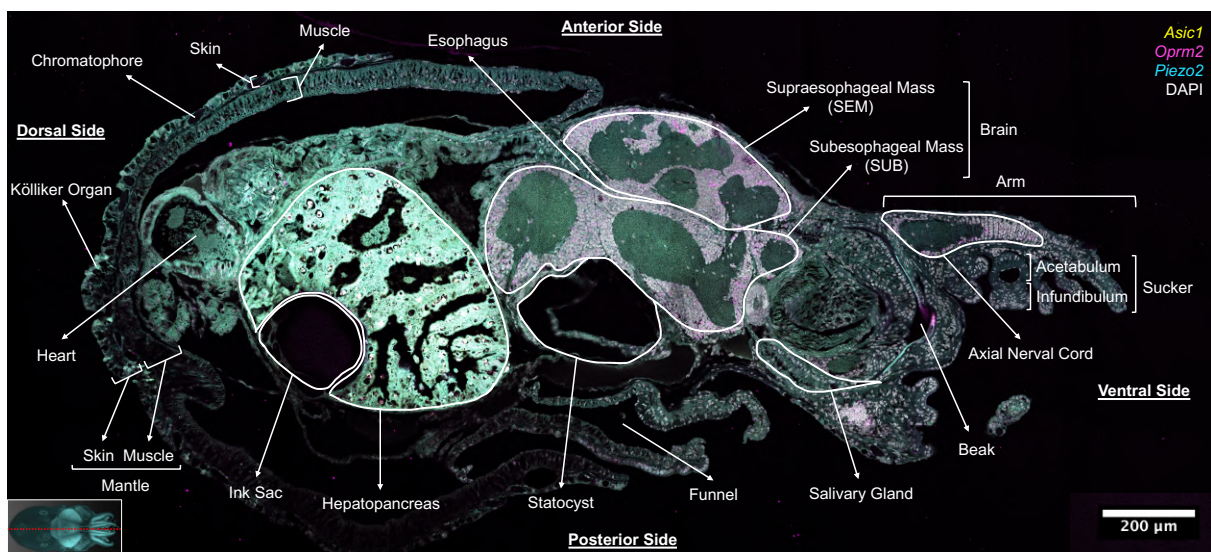


Figure 16: Overview of *Octopus vulgaris* paralarvae structures at 40 days post-hatching (dph). This overview figure shows the expression patterns of *Asic1* (yellow), *Oprm2* (pink), and *Piezo2* (blue), detected using hybridization chain reaction (HCR), and visualized by laser scanning confocal microscopy. DAPI (white) was used as a nuclear counterstain. Key anatomical structures for this thesis are labeled, including those potentially involved in nociception and pain-related gene expression. Directional indicators—anterior, posterior, dorsal, and ventral sides—are included to aid in spatial orientation. The inset in the bottom left corner illustrates the cutting plane used to obtain the section shown in this figure, providing additional context for anatomical interpretation. The scale bar represents 200 μm in actual size.

Figure 17 shows the expression patterns of *Asic1*, *Oprm2*, and *Ano1/2*, detected using HCR and visualized by laser scanning confocal microscopy. Again, DAPI was used as a nuclear counterstain. The paralarval section visualized in this figure was obtained using a different cutting plane compared to Figure 16. Therefore, Figure 17 further complements the anatomical context by depicting additional key structures relevant to this thesis, namely the optic lobe and stellate ganglion, which are not visible in Figure 16.

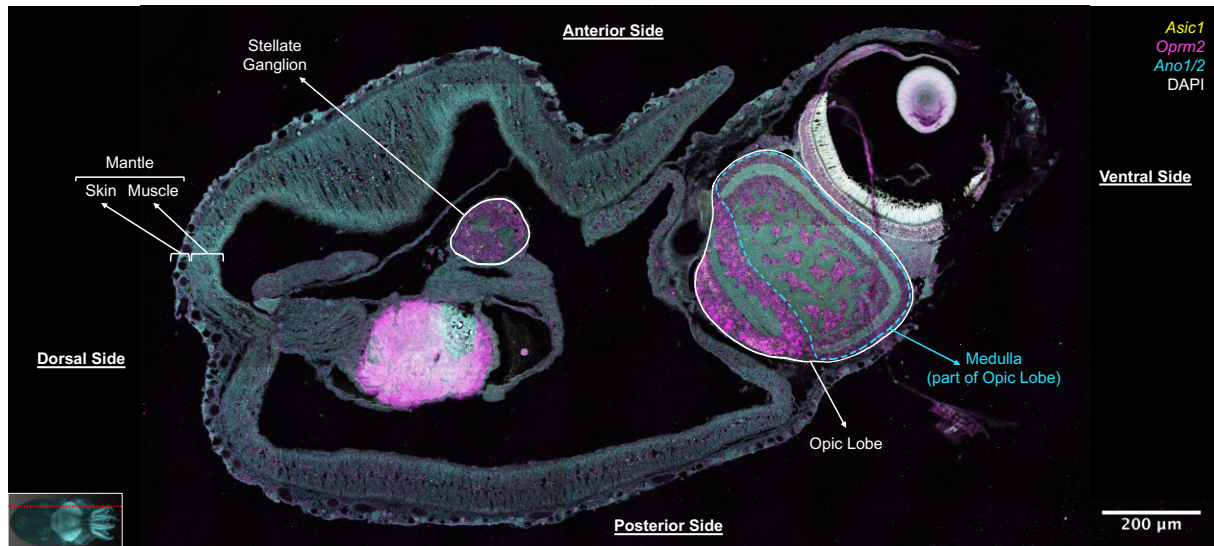


Figure 17: Additional overview of *Octopus vulgaris* paralarvae structures at 40 days post-hatching (dph). This overview figure shows the expression patterns of *Asic1* (yellow), *Oprm2* (pink), and *Ano1/2* (blue), detected using hybridization chain reaction (HCR), and visualized by laser scanning confocal microscopy. DAPI (white) was used as a nuclear counterstain. Key anatomical structures are labeled, including regions of interest for this thesis, such as the stellate ganglion, mantle, and the optic lobe, which are potentially involved in nociception and pain-related gene expression. Directional indicators—anterior, posterior, dorsal, and ventral sides—are included to aid spatial orientation. The inset in the bottom left corner illustrates the cutting plane used to obtain this section, providing context for anatomical interpretation. The scale bar represents 200 μm in actual size.

Now that the anatomical context is established, the next step is to investigate the expression patterns of the selected candidate genes potentially related to nociception. Table 2 (page 40) summarizes the expression patterns of *Kcnk1*, *Trpa1*, *Asic1*, *Ano1/2*, *Oprm2*, and *Piezo2* observed in *O. vulgaris* paralarvae at both 5 and 40 days post-hatching.

At 5 days post-hatching, *Kcnk1* showed very strong expression in the supraesophageal mass (SEM), especially in the basal lobe. Additionally, strong expression was observed in the buccal lobe and subesophageal mass (SUB), while weaker expression was present in the axial nerve cord and the optic lobe. At 40 days post-hatching, the expression pattern was largely maintained, with very strong expression continuing in the SEM, specifically in the basal lobe. At this stage, very strong expression was now also seen in the SUB and buccal lobe. Strong expression was observed in the axial nerve cord and the salivary gland, while weak expression appeared in the sucker. The expression in the stellate ganglion remains unknown for both developmental stages, as this structure was not visible in any of the available HCR sections targeting this gene. The expression patterns of *Kcnk1* are shown in Figure A1 (Addendum; pages A.14 – A.15).

For *Trpa1*, very strong expression was observed in the infundibulum of the sucker at both 5 dph and 40 dph. At 5 dph, weak expression was observed in the esophagus, but this was no longer present at 40 dph. At this later stage, strong expression appeared as a distinct line in the muscle of the mantle. The expression in the stellate ganglion remains unknown for both developmental stages, as this structure was not visible in any of the available HCR sections targeting this gene. The expression patterns of *Trpa1* are shown in Figure A2 (Addendum; pages A.16 – A.17).

Ano1/2 exhibited consistently very weak expression in the SEM, SUB, axial nerve cord, and sucker at both 5 and 40 dph. At 5 dph, strong expression was observed in the skin of the mantle and the funnel, while very weak expression was also noted in the salivary gland. By 40 dph, expression in the funnel and mantle persisted, though expression in the mantle had shifted from the skin to the underlying muscle layer. At this later stage, no expression was detected in the salivary gland. The expression of *Ano1/2* in the stellate ganglion remains undetermined at 5 dph, as this structure was not visible in any of the available HCR sections targeting this gene at 5 dph. At 40 dph, the stellate ganglion was visualized, but no *Ano1/2* expression was detected. The expression patterns of *Ano1/2* are illustrated in Figure A3 (Addendum; pages A.18 – A.19). For some structures, including the salivary gland at 5 dph, the SEM, SUB, and axial nerve cord at 40 dph, and the sucker at both stages, the expression signals were too weak to clearly visualize in the figure.

At 5 dph, *Asic1* showed weak expression in the SEM, SUB, and sucker, and very weak expression in the axial nerve cord. At this stage, expression in the optic lobe and stellate ganglion remains unknown, as these structures were not visible in any of the available HCR sections. At 40 dph, expression became strong in the SEM, SUB, buccal lobe, and funnel. Very strong expression was observed in optic lobe, including the medulla, and in the stellate ganglion, while weak expression persisted in the axial nerve cord. The expression patterns of *Asic1* are shown in Figure A4 (Addendum; pages A.20 – A.21).

At 5 dph, *Oprm2* showed strong expression in the SEM, the buccal lobe, and SUB. Very strong expression was observed in the infundibulum of the sucker, and weak expression was observed in the mantle. The expression in the optic lobe and stellate ganglion remains unknown at this stage, as these structures were not visible in any of the available HCR sections targeting this gene at 5 dph. At 40 dph, very strong expression was observed in the SEM, SUB, buccal lobe, stellate ganglion, and optic lobe, including the medulla. The sucker maintained strong expression in the infundibulum, although slightly reduced compared to 5 dph. Additionally, strong expression was observed in the axial nerve cord. The expression patterns of *Oprm2* are shown in Figure A5 (Addendum; pages A.22 – A.23).

Finally, *Piezo2* showed weak expression at 5 dph in the SEM, the paliovisceral lobe of the SUB, the sucker, and the mantle. The expression in the stellate ganglion remains unknown for both 5 and 40 dph, as this structure was not visible in any of the available HCR sections targeting this gene. At 40 dph, strong expression was present in the SEM, paliovisceral lobe of the SUB, the muscle of the mantle, the end of the funnel, the esophagus, and the rest of the gastrointestinal tract. Very strong expression was seen in the salivary gland. Additionally, weak expression was observed in the sucker, and very weak expression in the optic lobe. The expression patterns of *Piezo2* are shown in Figure A6 (Addendum; pages A.24 – A.25).

Table 2: Overview of gene expression patterns in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph), detected by HCR. For each gene—*Kcnk1*, *Trpa1*, *Ano1/2*, *Asic1*, *Oprm2*, and *Piezo2*—expression strength is indicated across various anatomical structures for both 5 dph and 40 dph. Expression intensity is categorized as follows: ++ = very strong expression, + = strong expression, – = weak expression, – – = very weak expression, and 0 = no expression. A question mark (?) indicates that the structure has not been observed for this gene, and the expression pattern is still unknown. Abbreviations: ANC = Axial Nerve Cord, SEM = Supraesophageal Mass, SUB = Subesophageal Mass, Ba L = Basal Lobe, Bu L = Buccal Lobe, Infund. = Infundibulum, PVL = Palliovisceral Lobe, GI Tract = Gastrointestinal Tract. “End” refers to the expression observed at the funnel's end.

Gene	Developmental Stage	Expression Strenght										
		SEM	SUB	ANC	Sucker	Mantle	Funnel	Salivary Gland	Esophagus	GI Tract	Optic Lobe	Stellate Ganglion
<i>Kcnk1</i>	5 dph	++ Ba L + Bu L	+	–	0	0	0	0	0	0	–	?
	40 dph	++ Ba L ++ Bu L	++	+	–	0	0	+	0	0	0	?
<i>Trpa1</i>	5 dph	0	0	0	++ Infund.	0	0	0	–	0	0	?
	40 dph	0	0	0	++ Infund.	+ Muscle	0	0	0	0	0	?
<i>Ano1/2</i>	5 dph	– –	– –	– –	– –	+ Skin	+	– –	0	0	0	?
	40 dph	– –	– –	– –	– –	+ Muscle	+	0	0	0	0	0
<i>Asic1</i>	5 dph	–	–	– –	–	0	0	0	0	0	?	?
	40 dph	+ Bu L	+	–	0	0	+	0	0	0	++ Medulla	++
<i>Oprm2</i>	5 dph	+ Bu L	+	0	++ Infund.	–	0	0	0	0	?	?
	40 dph	++ Bu L	++	+	+ Infund.	0	0	0	0	0	++ Medulla	++
<i>Piezo2</i>	5 dph	–	– PVL	0	–	–	0	0	0	0	0	?
	40 dph	+	+ PVL	0	–	+ Muscle	+ End	++	+	+	– –	?

5B. Discussion

5B.1. Limitations of the Study Related to Paralarval Rearing and Imaging

It is important to note that all paralarvae used in this study were raised in the laboratory and, while this provides a high degree of control over environmental variables, it also introduces limitations in terms of ecological validity. Lab-reared *Octopus vulgaris* paralarvae often exhibit high mortality rates and suboptimal growth, failing to reach the size or developmental potential observed in wild populations (Iglesias et al., 2007; Iglesias & Fuentes, 2014). These challenges have been attributed to multiple factors, including inadequate nutrition, poor water quality, and the absence of standardised culture protocols (Vaz-Pires et al., 2004; Iglesias et al., 2007; Navarro et al., 2014; Birch et al., 2021). Captive rearing is further complicated by the species' demanding dietary requirements; paralarvae rely on high densities of appropriate live prey, such as larval shrimp and enriched *Artemia*, to support their rapid growth and active jet-propelled movement (Villanueva et al., 1995, 1997; Mather & Scheel, 2014). While studies have shown that the use of specific prey sizes and enrichment strategies can improve survival and growth (Fuentes et al., 2009; Nabhitabhata & Segawa, 2014), achieving successful juvenile settlement remains rare and inconsistent (Iglesias & Fuentes, 2014). Consequently, artificial rearing conditions may significantly influence gene expression, stress physiology, and developmental outcomes. Therefore, while the imaging and gene expression patterns observed at 5 and 40 days post-hatching (dph) provide valuable insight into early developmental dynamics under controlled conditions, these findings cannot be directly extrapolated to wild populations.

Beyond the biological limitations imposed by artificial rearing in the laboratory, methodological constraints related to imaging must also be considered. In this study, laser scanning confocal microscopy (LSCM) was employed to visualize gene expression patterns in *Octopus vulgaris* paralarvae. A particular limitation of LSCM in the context of this study is that it ultimately provides two-dimensional views of individual optical sections. For this thesis, conclusions regarding gene expression patterns were based on the interpretation of selected single optical planes from confocal z-stacks. For complex three-dimensional structures, such as the suckers of *O. vulgaris* paralarvae, this complicates the precise determination of gene expression localization within the full 3D volume (Pawley, 2006; Elliott, 2020). For instance, while *Trpa1* expression was clearly detectable in the infundibulum of the sucker, its precise spatial localization within the sucker structure (e.g., superficial versus deep layers) cannot be determined from a single optical plane alone. Determining the exact 3D position of expression would require analyzing the entire z-stack or generating a reconstructed 3D volume. To overcome this limitation, future research should focus on such volumetric analyses to more confidently identify the spatial distribution of expression, and thus the potential localization of nociceptors, within the tissue.

5B.2. Identifying Candidate Genes Implicated in Nociception

The selection of candidate genes for the present study was mainly inspired by the doctoral research of Giulia Di Cristina, titled “Nociception in the cephalopod mollusc *Octopus vulgaris*: a contribution to mapping putative nociceptors in the octopus arm” (Di Cristina, 2017). Her work focused on adult *O. vulgaris* and employed a combination of transcriptome analysis (bioinformatics), gene expression studies (qPCR), and immunohistochemistry (IHC), intending to identify putative nociceptors and possible pathways within the octopus arm (Di Cristina, 2017). Table 3 of Di Cristina (2017) provided a list of 32 “pain” related genes from the *O. vulgaris* transcriptome. From this curated list, five genes were selected to investigate nociception in *O. vulgaris* paralarvae as part of this present study: *Trpa1*, *Asic1*, *Ano1*, *Oprm1*, and *Piezo2*. These genes were chosen due to their established or suspected involvement in nociceptive pathways. Some, such as *Oprm1* and *Trpa1*, were annotated with the Gene Ontology term “sensory perception of pain” (GO:0019233), while the remaining candidates had been previously linked to nociceptive processes based on evidence from various studies (Di Cristina, 2017). In addition to the five genes derived from Di Cristina’s thesis, a sixth gene, *Kcnk1*, was included in this study based on available gene expression data from the single-nucleus RNA sequencing (snRNAseq) dataset of 1 dph *Octopus vulgaris* provided by the Seuntjens Lab (KU Leuven). Importantly, due to some practical considerations related to probe design and gene expression, some of the initially intended gene targets required substitution or adjustment. Most notably, *Oprm2* was selected in place of *Oprm1* because the mRNA sequence of *Oprm1* was too short to design a reliable HCR probe. Similarly, the designation *Ano1/2* was used instead of *Ano1*, as the limited resolution of available transcriptome annotations did not allow for a clear distinction between *Ano1* and *Ano2* transcripts. These practical limitations shaped the final gene panel used in this thesis to be *Kcnk1*, *Trpa1*, *Asic1*, *Ano1/2*, *Oprm2*, and *Piezo2*.

5B.3. Comparative Analysis of Expected and Observed Gene Expression

Where relevant, findings from Di Cristina’s doctoral research will be used to contextualize and compare the results presented in this present study. Importantly, while both this study and the doctoral thesis of Di Cristina share a thematic focus on nociception, they differ substantially in both methodology and developmental scope. Di Cristina (2017) examined gene expression in adult *O. vulgaris* using quantitative PCR (qPCR) and immunohistochemistry (IHC). In contrast, this study investigated gene expression at earlier developmental stages of *O. vulgaris* (5 and 40 dph paralarvae) using hybridization chain reaction (HCR). Both HCR and qPCR detect mRNA, but they provide different types of information. HCR is a qualitative technique that shows where genes are being actively transcribed, offering spatial resolution of mRNA expression (Choi et al., 2018; Schwarzkopf et al., 2021). qPCR, by contrast, quantifies the amount of specific RNA transcripts in a sample, providing a measure of gene expression levels but no information about spatial localization (Thermo Fisher Scientific, n.d.).

Similar to HCR, IHC also provides spatial information, but it differs fundamentally from HCR in that it visualizes gene products (proteins) rather than mRNA, thereby offering insight into where proteins are present and likely functional (Anderson & Westra, 2025).

When comparing findings, these differences should be taken into account for several reasons. First, mRNA may be synthesized in one region, while the corresponding protein is transported elsewhere to carry out its function (Alberts et al., 2002). As a result, the location where the gene is transcribed might not match where the protein ends up. Second, mRNA and protein presence can vary significantly across developmental stages, meaning that a gene active in adults may not yet be expressed, or expressed differently, at earlier stages such as 5 or 40 dph paralarvae. These differences make direct comparisons challenging, but not impossible. Meaningful insights can still be drawn by examining whether transcriptional activity at early stages aligns with known gene and protein localization in adults, and whether expression patterns change or emerge gradually during development.

5B.3.1. *Kcnk1* – Potassium Channel Subfamily K Member 1

Kcnk1, encoding the protein TWIK-1 (Two-Pore domain Weak Inwardly rectifying K⁺ channel 1), belongs to the two-pore domain potassium (K2P) channel family, which is well-characterized in vertebrates (Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015; Ben Soussia et al., 2019; Luo et al., 2021). In these systems, K2P channels are essential for maintaining resting membrane potential and regulating neuronal excitability, functions that are fundamental to nervous system physiology and likely conserved across diverse phyla (Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015; Ben Soussia et al., 2019). Given the remarkable complexity of the cephalopod nervous system (Mather & Dickel, 2017; Ponte et al., 2021; Albertin & Katz, 2023), it is plausible that K2P channels, including *Kcnk1*, are also functionally relevant in these animals, even if they remain uncharacterized.

The expression pattern of *Kcnk1* observed in this study aligns with its known role in maintaining resting membrane potential and regulating neuronal excitability, as described in vertebrate systems (Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015; Ben Soussia et al., 2019). At both 5 and 40 dph, strong expression was detected in the SEM, particularly in the basal lobe, a region associated with integrative functions and motor coordination in cephalopods (Mather & Dickel, 2017; Ponte et al., 2021; Albertin & Katz, 2023). This expression pattern suggests that *Kcnk1* contributes to foundational neural functions early in development, likely supporting central processing and behavioral control. Notably, strong expression was also observed in the buccal lobe and SUB, with intensity increasing at 40 dph. These areas are involved in feeding and arm control, and their developmental upregulation may reflect the functional maturation of motor circuits. This is consistent with the broader role of K2P channels in modulating excitability across central systems (Lesage & Lazdunski, 2000; Mathie & Veale, 2015).

In addition to its central expression, *Kcnk1* was also detected in peripheral and autonomic-related structures. At 5 dph, weak expression was observed in the axial nerve cord, which became slightly stronger by 40 dph, suggesting an increasing role in peripheral excitability regulation as the animal matures. By 40 dph, expression also appeared in the salivary gland and sucker, regions likely associated with emerging secretory function and refined sensorimotor control, respectively. These developmental changes point to an expanding functional repertoire for *Kcnk1*, potentially supporting the growing behavioral and physiological complexity of juvenile *O. vulgaris*. While *Kcnk1* is not typically classified as a canonical nociceptor gene, it plays a well-documented modulatory role in nociception by fine-tuning neuronal excitability and pain sensitivity (Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015; Ben Soussia et al., 2019). These processes are central in pain modulation, chronic sensitization, and potentially even the subjective experience of pain (Lesage & Lazdunski, 2000; Honoré, 2007; Mathie & Veale, 2015; Ben Soussia et al., 2019). The strong and consistent expression of *Kcnk1* in central integrative regions, combined with its expanding expression in peripheral and autonomic structures between 5 and 40 days post-hatching, suggests a developmentally sustained and functionally broad role in the *O. vulgaris* nervous system. Its distribution across regions associated with sensorimotor integration and behavioral coordination points to a potential involvement in shaping excitability thresholds during development.

5B.3.2. *Trpa1* – Transient Receptor Potential Cation Channel Subfamily A Member 1

Trpa1 encodes a non-selective cation channel (TRPA1) known for its broad chemical sensitivity and its involvement in nociception (Di Cristina, 2017). The TRPA1 channel can be activated by a variety of exogenous and endogenous compounds, including bradykinin, formalin, anandamide, tetrahydrocannabinol, and reactive electrophiles such as allyl isothiocyanate (AITC), a component of mustard oil (Bandell et al., 2004; Di Cristina, 2017). TRPA1 may also have thermosensitive properties, but whether it is directly activated by cold temperatures below 17 °C or instead contributes to cold hypersensitivity remains under debate (Laursen et al., 2015). Some authors have suggested that TRPA1 may serve as a central component of chemically induced pain signaling (Tai et al., 2008). In support of this role, several studies have examined its distribution across nociceptor subtypes in vertebrates. While some report predominant co-expression of TRPA1 with calcitonin gene-related peptide (CGRP) (Bautista et al., 2005), a marker of peptidergic nociceptors, others have found that over 90% of TRPA1-positive neurons are co-labeled with Isolectin B4 and can therefore be classified as non-peptidergic nociceptors (Barabas et al., 2012; Di Cristina, 2017). Interestingly, people with elevated pain sensitivity show differential DNA methylation near the *TRPA1* gene, suggesting that epigenetic variation may contribute to individual differences in pain sensitivity (Di Crisina, 2017).

In addition to vertebrates, *Trpa1* expression has also been studied in a range of invertebrates (Di Cristina, 2017). TRPA1 homologs have been identified in *Drosophila melanogaster*, where the homologous gene *painless* is essential for detecting noxious heat in larvae (Tracey et al., 2003), as well as in *Caenorhabditis elegans*, *Nematostella vectensis*, *Crassostrea gigas*, and *Octopus bimaculoides* (Di Cristina, 2017).

In this study, *Trpa1* exhibited strong and spatially distinct expression in *O. vulgaris* paralarvae, with consistently high levels observed in the infundibulum of the sucker at both 5 and 40 dph. This region is known to be densely innervated and functionally specialized (Young 1965; Di Cristina, 2017). Notably, Di Cristina (2017) also reported the presence of TRPA1 within the epithelium covering the infundibulum of adult *O. vulgaris* suckers, supporting a sensory role for this region. These findings collectively support the interpretation that *Trpa1* expression in the sucker may reflect an early role in peripheral sensory signaling. Additionally, according to Di Cristina (2017), *Trpa1* expression in adult *O. vulgaris* also appears to be (not significantly) higher in the optic lobe compared to other tissues. In the present study, however, *Trpa1* expression was not detected in the optic lobe at either 5 or 40 dph, suggesting that expression in this region emerges later in development. During this study, developmental differences in *Trpa1* expression were also observed between 5 and 40 dph. At 5 dph, weak expression was detected in the esophagus, but this signal was no longer present at 40 dph, suggesting a transient developmental role. Conversely, by 40 dph, *Trpa1* expression emerged as a distinct linear pattern within the muscle of the mantle, a pattern not present at 5 dph, which may indicate a developmental shift toward involvement in motor or mechanosensory processes.

5B.3.3. *Ano1/2* – Anoctamin-1 and -2

The genes *Ano1* and *Ano2* belong to the anoctamin family (also known as the TMEM16 family), which comprises ten genes in total (Di Cristina, 2017). Specifically, *Ano1* encodes the protein ANO1 (TMEM16A) and *Ano2* encodes the protein ANO2 (TMEM16B) (Schroeder et al., 2008; Di Cristina, 2017; DiBattista et al., 2024). Among the members of the TMEM16 family, the proteins ANO1 and ANO2 are most similar, sharing about 60% amino acid identity (Yang et al., 2008), and both function as calcium-activated chloride (Cl⁻) channels (Di Cristina, 2017; DiBattista et al., 2024). *Ano1* influences how cells respond to various stimuli in smooth muscles, the heart, the endothelium, neuronal tissues, and epithelial organs (Kunzelmann et al., 2012). In mice, *Ano1* is highly expressed in dorsal root ganglion neurons, where its product, ANO1, co-localizes with *TRPV1*, a well-known nociceptive marker (Cho et al., 2012; Lee et al., 2014). Functional ablation studies in mice have shown that loss of *ANO1* in these neurons results in reduced thermal pain sensitivity, suggesting a direct role in nociceptive processing (Cho et al., 2012; Cho & Oh, 2013; Lee et al., 2014). Additionally, *ANO1* contributes to inflammation-induced increases in neuronal excitability, likely through phosphorylation-dependent modulation by various kinases, further implicating it in the sensitization of nociceptors (Lee et al., 2014).

Similarly, *Ano2* is primarily expressed in neurons, where it plays a key role in modulating neuronal excitability (Schreiber et al., 2010; DiBattista et al., 2024). In vertebrates, ANO2 has been shown to reduce neuronal excitability in the hippocampus (Huang et al., 2012), thalamus (Ha et al., 2016), lateral septum (Wang et al., 2019), and central lateral amygdala (Li et al., 2019), whereas it increases excitability in olfactory receptor neurons (Billig et al., 2011; Pietra et al., 2016), and within the olivo-cerebellar system (Zhang et al., 2015; Zhang et al., 2017; Auer et al., 2021). In olfactory sensory neurons, ANO2 contributes to amplification of the odorant response and modulates neuronal firing, response kinetics, and adaptation (DiBattista et al., 2024). While the presence of anoctamin genes in invertebrates has not yet been extensively characterized (Di Cristina, 2017), genomic analyses suggest that invertebrate species may possess a diverse and complex repertoire of anoctamin paralogs (Milenkovic et al., 2010; Di Cristina, 2017). For this thesis, *Ano1/2* was selected as a candidate gene to investigate nociception in *O. vulgaris* paralarvae. As octopuses did not undergo a whole-genome duplication (Albertin et al., 2015), distinct *Ano1* and *Ano2* paralogs are not present in cephalopods. As a result, the target was designated *Ano1/2*.

In this study, *Ano1/2* exhibited weak and spatially restricted expression patterns across most examined tissues, with some notable developmental dynamics between 5 and 40 dph. At 5 dph, expression was strongest in the skin of the mantle and funnel, suggesting an early role at the interface between the organism and its environment. By 40 dph, expression in the funnel was maintained, and expression in the mantle shifted from the skin to the underlying muscle layer, suggesting a developmental transition from superficial epithelial roles to deeper neuromuscular functions. This pattern aligns with vertebrate studies showing that ANO1, the protein product of *Ano1*, contributes to both smooth muscle excitability and epithelial ion transport (Kunzelmann et al., 2012; Di Cristina, 2017), and supports the involvement of TMEM16-family chloride channels in sensory and contractile modulation during early cephalopod neurodevelopment. In contrast, expression in central nervous system-associated regions such as the SEM, SUB, axial nerve cord, and sucker remained very weak at both 5 and 40 dph. This pattern diverges from vertebrate models, where ANO1 and ANO2 are robustly expressed in sensory and central neurons (Cho et al., 2012; Lee et al., 2014; Auer et al., 2021; DiBattista et al., 2024). However, it is also possible that the expression of these channels in the cephalopod nervous system occurs later in development, beyond the paralarval stages examined here. The observed discrepancy may reflect species-specific differences in gene function or timing of expression. Alternatively, it could be due to detection limitations, particularly given the incomplete annotation of *O. vulgaris* transcripts, which required the use of a combined probe targeting *Ano1/2*. Additionally, expression in the salivary gland was very weak at 5 dph and undetectable at 40 dph, suggesting transient developmental expression.

5B.3.4. *Asic1* – Acid-Sensing Ion Channel Subunit 1

Acid-sensing ion channels (ASICs) are a class of pH-sensitive receptors that generate acid-gated currents and are closely related to the degenerin-epithelial sodium (Na⁺) channel (DEG/ENaC) family (Waldmann et al., 1997; Mano & Driscoll, 1999; Di Cristina, 2017). These channels are widely diffused in both central and peripheral neurons and have been implicated in a variety of cellular functions (Di Cristina, 2017). In sensory nerve terminals, ASICs are believed to play a significant role in detecting painful or mechanical stimuli (Price et al., 2001), although their precise activation mechanisms within the brain remain poorly understood (Di Cristina, 2017). ASIC function is influenced by a diverse set of endogenous and exogenous modulators, including dynorphin, FMRFamide, and the pharmacological agent amiloride (Wemmie et al., 2013; Di Cristina, 2017). Despite their known roles in vertebrates (Alvarez de la Rosa et al., 2003; Omerbašić et al., 2015), very little is known about the presence of these channels in molluscs and invertebrates in general (Di Cristina, 2017). Nevertheless, putative orthologs of *asic1* have been identified in invertebrate species such as *C. elegans* and *Octopus bimaculoides* (Albertin et al., 2015). Additionally, the presence of *Asic1* transcripts in *O. vulgaris* was confirmed based on transcriptome data (Petrosino, 2015). Expression analysis in *O. vulgaris* suggests that *Asic1* is most abundantly expressed in the tip of the arm and subesophageal mass (SUB), although no statistically significant differences in expression levels were observed between these regions (Di Cristina, 2017).

In this study, *Asic1* expression exhibited clear developmental changes between 5 and 40 dph, characterized by both spatial expansion and increased signal intensity. At 5 dph, weak expression was observed in the SEM, SUB, and sucker, with very weak signal in the axial nerve cord. By 40 dph, expression intensified in the SEM and SUB and was newly detected in the buccal lobe and funnel. Very strong expression emerged in the optic lobe, including the medulla, and in the stellate ganglion, structures not visible in earlier sections. These patterns point to a developmentally regulated expression profile, with *Asic1* becoming more broadly involved in neural processing as the nervous system matures. The enhanced expression in known integrative and motor control centers, such as the SEM, SUB, buccal lobe, and funnel, supports a role for *Asic1* in the maturation of sensorimotor coordination. The early expression in the sucker may reflect a transient peripheral sensory role, consistent with ASIC function in vertebrate sensory terminals (Price et al., 2001; Wemmie et al., 2013), while its absence at 40 dph could indicate either a developmental shift or spatial reallocation of function. Notably, the strong expression at 40 dph in the optic lobe and stellate ganglion, regions involved in visual processing and mantle motor control, respectively, suggests that *Asic1* may support the emergence of more complex behaviors requiring visual-motor integration.

Compared to transcriptomic findings in adult *O. vulgaris*, which reported *Asic1* expression in the SUB and arm tip (Petrosino, 2015; Di Cristina, 2017), the present study provides a broader anatomical perspective. While there is some overlap in the SUB, the additional expression detected in regions such as the optic lobe and stellate ganglion suggests that *Asic1* activity extends well beyond the arm and is already established during early post-hatching stages. These findings highlight the importance of spatially resolved developmental studies and suggest that ASIC-related signaling may play a central role in shaping sensorimotor networks from a young age.

5B.3.5. *Oprm2* – Opioid Receptor μ -type 2

Five opioid receptor types have been identified to date, classified based on the ligand and effects at cellular level: μ (mu) receptors (MOR or OPRM), κ (kappa) receptors (KOR), δ (delta) receptors (DOR), nociceptin/orphanin FQ receptors (NOR), and ζ (zeta) receptors (ZOR) (Di Cristina, 2017; Dhaliwal & Gupta, 2023). These receptor classes encompass several subtypes, including $\mu 1$, $\mu 2$, and $\mu 3$, $\kappa 1$, $\kappa 2$, and $\kappa 3$, and $\delta 1$ and $\delta 2$ (Dhaliwal & Gupta, 2023). All types are G protein-coupled receptors (GPCRs) that respond to both endogenous peptides and exogenous compounds (Di Cristina, 2017; Dhaliwal & Gupta, 2023). In vertebrates, opioid receptors are densely distributed in key regions of the nervous system, including the periaqueductal gray, locus coeruleus (LC), rostral ventral medulla, dorsal horn of the spinal cord (particularly the substantia gelatinosa), and peripheral afferent nerves (Dhaliwal & Gupta, 2023). Peripheral receptors detect painful stimuli and relay signals via the spinal cord to the brain (Dhaliwal & Gupta, 2023). Activation of μ -type opioid receptors (MOR/OPRM) by an opioid agonist in the midbrain, mediated through inhibition of gamma-aminobutyric acid (GABA) interneurons, causes descending inhibitory impulses in the periaqueductal gray (Dhaliwal & Gupta, 2023). The periaqueductal gray stimulates descending inhibitory neurons (Toubia & Khalife, 2019), which activate enkephalin-containing neurons that are connected directly with the dorsal horn and subsequently lead to a decrease in nociceptive transmission from the periphery to the thalamus (Dhaliwal & Gupta, 2023). OPRMs also tone down the central stress response by inhibiting norepinephrine (NE) secretion from the LC, counteracting stress-induced NE release triggered by corticotropin-releasing hormone (CRH) from the hypothalamus (Dhaliwal & Gupta, 2023). Therefore, μ -type opioid receptors play a key role in stress recovery, as evidenced by studies showing that morphine administration following a traumatic event can reduce the risk of developing post-traumatic stress disorder (PTSD) (Toubia & Khalife, 2019). Additionally, high OPRM expression in the limbic system, an area involved in emotional regulation, suggests a role in mood modulation (Dhaliwal & Gupta, 2023). This makes these receptors also promising targets for the treatment of mood disorders such as anxiety and depression (Dhaliwal & Gupta, 2023). Lastly, the reward center of the brain involves the ventral tegmental area (VTA), which sends projections to the ventral striatum within the mesolimbic dopamine system. Stimulation of OPRMs by inhibiting GABA secretion results in increased dopamine release. Dopamine mediates the rewarding effects of opioid administration, thereby contributing to positive reinforcement (Toubia & Khalife, 2019).

Di Cristina (2017) reported elevated expression levels of *Oprm1* in adult *Octopus vulgaris*, with the highest levels detected in the optic lobe, followed by the tip of the arm. In contrast, significantly lower expression was observed in the SUB and other brain regions (Di Cristina, 2017). These findings suggest a potential central role for opioid signaling in the visual and integrative centers of the octopus brain and support continued investigation into its function as a modulator of nociceptive sensitivity in cephalopods (Di Cristina, 2017). Although *Oprm1* was originally selected for investigation in this study, technical limitations related to the short mRNA sequence prevented the design of reliable HCR probes. As a result, *Oprm2* was selected as an alternative target for gene expression analysis. However, while *Oprm1* expression has been characterized in adult *O. vulgaris*, no comparable data are currently available for *Oprm2*.

In this study, *Oprm2* expression showed a clear developmental progression in *O. vulgaris* paralarvae, with signal strength increasing and distribution broadening between 5 and 40 dph. At 5 dph, expression was already strong in central motor and integrative regions such as the SEM, SUB, and buccal lobe, and very strong in the infundibulum of the sucker, a structure previously identified as a sensory site in adult *O. vulgaris* (Di Cristina, 2017). By 40 dph, *Oprm2* expression intensified in the SEM and SUB and expanded to include very strong signals in the optic lobe and stellate ganglion, as well as strong expression in the axial nerve cord. The high expression levels in regions associated with sensorimotor coordination and peripheral input, particularly the buccal lobe, axial nerve cord, sucker, and stellate ganglion, suggest a role for *Oprm2* in modulating both central and peripheral neural activity. This pattern aligns with the established function of μ -type opioid receptors in vertebrates, where they inhibit nociceptive transmission and regulate stress and affective responses through descending control pathways (Dhaliwal & Gupta, 2023; Toubia & Khalife, 2019). Although no published data are currently available on *Oprm2* expression in adult *O. vulgaris*, the strong signals observed at 40 dph, particularly in the optic lobe and other central brain regions, suggest that opioid signaling involving this receptor is already active during early developmental stages and may contribute to the maturation of sensory and integrative networks.

5B.3.6. *Piezo2* – Piezo-type mechanosensitive ion channel component 2

Piezo2 is a rapidly adapting, trimeric, mechanically-gated ion channel found in a subset of sensory neurons within the mammalian dorsal root ganglion and in specialized cutaneous mechanoreceptors known as Merkel cell–neurite complexes (Coste et al., 2010; Woo et al., 2014; Sánchez-Carranza et al., 2024). Studies in mice have shown that deletion of *Piezo2* leads to a substantial loss of sensitivity to innocuous mechanical stimuli, while responses to noxious mechanical and thermal stimuli remain unaffected (Ranade et al., 2014). Conversely, in *Drosophila*, *Piezo* is required specifically for mechanical nociception, as knockout larvae exhibit strongly diminished responses to noxious mechanical stimuli but retain normal responses to gentle touch (Kim et al., 2012).

Additional research in mice by Sánchez-Carranza et al. (2024) examined sensory neurons from dorsal root ganglia using electrophysiology to assess mechanoreceptor and nociceptor function, alongside behavioral and morphological analyses. Mutations at the conserved Arginine (R2756) altered the biophysical properties of the PIEZO2 channel by relieving the voltage block and lowering the mechanical thresholds for channel activation (Sánchez-Carranza et al., 2024). Homozygous knock-in mice were viable and displayed a marked sensitization of mechanosensitive currents in nociceptors, whereas touch-related mechanoreceptors were only mildly affected (Sánchez-Carranza et al., 2024). These findings suggest that PIEZO2 plays a dynamic role in mechanical pain sensitivity: when the channel's voltage block is relieved, nociceptors can become increasingly sensitized (Sánchez-Carranza et al., 2024). As reported by Di Cristina (2017), differential expression analysis in *O. vulgaris* revealed that *Piezo2* is significantly more expressed in the subesophageal mass (SUB) than in other tissues, potentially pointing to a functional role in central mechanosensory processing.

In this study, *Piezo2* expression in *O. vulgaris* paralarvae exhibited clear developmental progression between 5 and 40 dph, characterized by both increased signal intensity and broader anatomical distribution. At 5 dph, expression was weak and limited to the SEM, SUB, specifically the palliovisceral lobe, the sucker, and mantle. By 40 dph, expression had intensified in the SEM and SUB and expanded to peripheral structures, including the mantle musculature, funnel, esophagus, gastrointestinal tract, and notably the salivary gland, which showed very strong expression. In contrast, only weak signal remained in the sucker and optic lobe. This spatial and temporal pattern suggests a developmentally regulated role for *Piezo2* in both central mechanosensory integration and peripheral mechanotransduction. The increased expression in visceral and musculature-associated tissues at 40 dph supports a potential role in functions such as locomotion, feeding, and possibly defensive behaviors. These findings are consistent with cross-species studies showing that PIEZO channels mediate different forms of mechanical sensitivity: in mice, *Piezo2* is required for innocuous touch but not mechanical pain (Ranade et al., 2014), while in *Drosophila*, *Piezo* is essential for detecting noxious mechanical stimuli, but dispensable for gentle touch sensation (Kim et al., 2012). Importantly, strong expression in the SUB, especially the palliovisceral lobe, supports earlier findings by Di Cristina (2017), who suggested a central role for *Piezo2* in mechanosensory processing in cephalopods. The current results extend this by demonstrating that such expression is already present in early paralarval stages and becomes more prominent over development.

6B. Conclusions and Future Perspectives

This study investigated the developmental expression of six genes associated with nociception and mechanosensory signaling in *Octopus vulgaris* paralarvae. The results show that components of conserved sensory and modulatory pathways are already active during early post-hatching stages and often exhibit region-specific and developmentally dynamic expression. These findings suggest that paralarvae possess a surprisingly mature molecular basis for sensory detection and modulation. *Asic1* was expressed beyond previously reported adult peripheral regions and showed increasing expression across development in central structures such as the optic lobe, stellate ganglion, and funnel. This pattern points to its involvement in shaping sensorimotor circuitry and suggests that ASIC-based excitability regulation may be active from an early stage. *Piezo2* also showed expression consistent with a role in the maturation of sensorimotor systems, suggesting it contributes to mechanosensory and potentially nociceptive signaling during development. *Oprm2* expression extended the known distribution of opioid receptor activity in cephalopods, indicating possible roles in sensory modulation, behavioral regulation, and stress processing, similar to its paralog *Oprm1*. *Trpa1* displayed sustained expression in the suckers and later emergence in the mantle at 40 dph, supporting early involvement in sensory function and a potential later role in motor or mechanosensory pathways. *Ano1/2* expression suggested the presence of anoctamin-like channels that are developmentally regulated, though the inability to distinguish between paralogs due to current annotation limitations highlights the need for improved transcriptomic resources. Finally, *Kcnk1* emerged as a promising gene related to the modulation of neuronal excitability thresholds, identifying it as a potential contributor to nociceptive sensitivity and a candidate for comparative studies on the evolution of pain-related pathways.

Collectively, these findings provide strong evidence that *O. vulgaris* paralarvae possess the molecular infrastructure required for nociception. However, while nociception is a necessary precondition for the possibility of pain, it is not sufficient on its own. A true pain experience involves conscious awareness of tissue damage and an associated negative emotional state. As such, it remains too early to conclude whether *O. vulgaris* paralarvae are capable of experiencing pain. The gene expression data suggest the presence of functional sensory pathways, but do not, on their own, confirm the existence of affective or conscious experience. Future studies should aim to validate gene function through techniques such as electrophysiology, calcium imaging, and gene knockdown. Behavioral assays will be necessary to assess whether paralarvae exhibit pain-like responses, such as sensitization or conditioned avoidance. Comparative and cross-stage studies could further clarify whether nociceptive pathways are conserved across cephalopods and to what extent these molecular findings correlate with actual sensory experience.

7. References

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8. Addendum

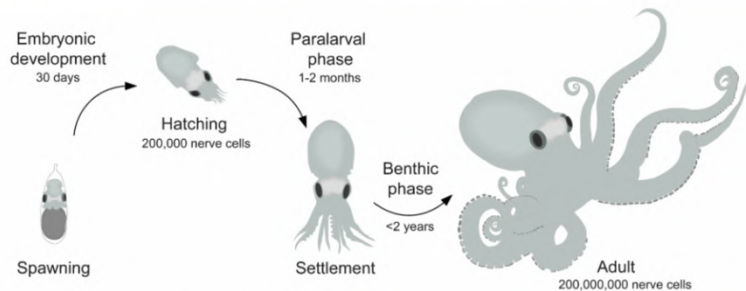
8.1. Supplementary Data

8.1.1. Template Survey – Exploring Perceptions and Reasoning in Octopus Research

Ethical considerations in animal research, focusing on *Octopus vulgaris*

Thank you for your interest in this research study!

This study aims to explore how people evaluate ethical considerations in scientific research involving animal models, focusing particularly on a model organism for brain research: the common octopus, *Octopus vulgaris*. Besides the adult octopus, this study will also focus on the paralarval stage of *O. vulgaris*. These 'paralarvae' hatch with about 1.0-1.5 mm of mantle length, are planktonic, swim actively, and have high metabolic rates. During the planktonic phase, the paralarvae undergo strong morphological changes and, as a result, the squid-like shape of hatchlings progressively merges to the octopus shape at settlement. The duration of the paralarval period is temperature-dependent and lasts around 47-54 days at 21.2°C and 30-35 days at 23°C, after which the octopuses start settling to the bottom.



For this survey, you will first answer some demographic questions, after which you will read a short abstract about *O. vulgaris* research and answer a series of questions about your ethical judgments and beliefs. The survey will take approximately 10 minutes to complete.

☐ I agree

[Show policy](#)

Privacy policy

Your participation is entirely voluntary. You may choose not to participate or withdraw from the study at any time, without giving a reason or facing any consequences.

Your responses in this study will be handled with the utmost care and remain strictly confidential. While the collected data could theoretically allow for identification, this is not our intention, and no effort will be made to trace individual participants. Furthermore, the results presented in any publications will be fully anonymized, ensuring that no individual can be identified.

There are no anticipated risks associated with your participation in this study.

While there are no direct benefits to you, your participation will contribute to a better understanding of beliefs about animal pain and their implications for science and ethics.

The data collected will be used solely for academic purposes and may be shared in publications or presentations. All data will be stored securely and handled in accordance with relevant privacy regulations.

If you have any questions about the study or your participation, please contact eve.seuntjens@kuleuven.be or andreas.deblock@kuleuven.be

By clicking "I agree" and proceeding to the survey, you confirm that:

- You have read and understood the information provided above.
- You voluntarily agree to participate in this study.
- You are 18 years or older.
- You know you will not receive any reward or compensation for your participation.

Accept

Close

Next

Cephalopod Biologists – Other Biologists

*To which group do you belong?

Choose one of the following answers

- or
- ☐ Biologist performing research using cephalopods (*Octopus vulgaris* and other species)
 - ☐ Biologist performing research using other (model) organisms
 - ☐ Non-biologist

Next

*How old are you?

🗳️ Choose one of the following answers

- ☐ 18 - 30 years old
- ☐ 31 - 40 years old
- ☐ 41 - 50 years old
- ☐ 50+ years old

Next

*What is your nationality?

🗳️ Choose one of the following answers

- ☐ American
- ☐ Belgian
- ☐ British
- ☐ Canadian
- ☐ Chinese
- ☐ Dutch
- ☐ French
- ☐ German
- ☐ Greek
- ☐ Indian
- ☐ Italian
- ☐ Japanese
- ☐ Mexican
- ☐ Nigerian
- ☐ Portuguese
- ☐ Russian
- ☐ Spanish
- ☐ Swedish
- ☐ Other:

Next

*How do you identify?

🗳️ Choose one of the following answers

- ☐ Male
- ☐ Female
- ☐ Non-binary
- ☐ I prefer not to answer this question
- ☐ Other:

Next

*What is your field of expertise?

Next

*How many years of academic experience do you have?

● Choose one of the following answers

☐ Less than 5 years of experience

☐ 5 - 10 years of experience

☐ 11 - 15 years of experience

☐ 16 - 20 years of experience

☐ More than 20 years of experience

Next

*What (model) organism(s) have you worked with and/or are you currently working with?

Next

*On a scale from 1 to 5, how familiar are you with animal research ethics?

● 1 = not familiar at all

5 = very familiar

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Have you conducted or supervised research involving *Octopus vulgaris* or similar species?

☒ Yes

☐ No

Next

In this next “question,” one of three different abstracts will appear: Scenario 1, Scenario 2, or Scenario 3. The abstract that appears is completely random and will change each time the survey is started.

Scenario 1:

*Read the following abstract:

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Recently, researchers studied the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris*. Analysis was conducted using state-of-the-art molecular biological techniques. They found evidence that these genes are expressed in the paralarval stage.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

Scenario 2:

*Read the following abstract:

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers at KU Leuven are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Researchers studied the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris* to get a better understanding of nociception in this stage. Analysis was conducted using state-of-the-art molecular biological techniques. They found no evidence of these genes being expressed in the paralarval stage.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

Scenario 3:

*Read the following abstract:

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers at KU Leuven are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Researchers plan to study the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris* to get a better understanding of nociception in this stage. Analysis will be conducted using state-of-the-art molecular biological techniques. The experiment has not yet been conducted.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

*Do you think paralarval *Octopus vulgaris* individuals experience pain?



Yes



No

*On a scale from 1 to 5, how confident are you in your interpretation of whether paralarval *Octopus vulgaris* individuals might experience pain or not?

☒ 1 = very uncertain

5 = very certain

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you believe more research is necessary to resolve this uncertainty concerning pain experience in *Octopus vulgaris* paralarvae?

Choose one of the following answers

- ☐ Yes
- ☐ No
- ☐ I do not believe there is uncertainty

Next

If the answer to the previous question was “yes”, the question underneath will be shown. If any of the other answer options are chosen, the question underneath will not be shown, and the survey will automatically skip to the next question.

*What type(s) of research would you recommend to resolve this uncertainty?

Some explanation about the techniques:

- **Behavioral studies:** Observing and analyzing how *Octopus vulgaris* behaves in different situations to understand its actions, learning, and problem-solving abilities.
- **Molecular neurobiological analyses:** Examining the brain and nervous system of *Octopus vulgaris* at the molecular level, such as studying genes, proteins, and cells to understand how its nervous system works.
- **Alternative methods:** Using lab-based techniques like studying octopus cells or tissues in a dish, or computer models, instead of experimenting on live animals.

Select all that apply

- ☐ Behavioral studies
- ☐ Molecular neurobiological analyses
- ☐ Alternative methods (e.g. in vitro studies excluding “real” animal models)
- ☐ Other:

Next

*On a scale from 1 to 5, how ethical do you find the use of *Octopus vulgaris* paralarvae in the context of neurobiological experiments?

The paralarvae are first sedated by exposing them to 2.5% ethanol and after they can be exposed to 4% ethanol to kill them. Dead paralarvae are used to make sections for studying gene expression.

1 = not ethical at all

5 = completely ethical

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate the societal relevance of neurobiological research on *Octopus vulgaris* paralarvae?

Research is societally relevant when it answers questions that society asks, or solves problems that society faces.

1 = not relevant at all

5 = highly relevant

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*To what extent do you agree with the following statement: "The societal relevance of this research ethically outweighs the potential pain experienced by paralarval *O. vulgaris*."

1 = strongly disagree
5 = strongly agree

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you think adult *Octopus vulgaris* individuals experience pain?

☒ Yes ☐ No

Next

*On a scale from 1 to 5, how confident are you in your interpretation of whether adult *Octopus vulgaris* individuals might experience pain or not?

1 = very uncertain
5 = very certain

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you believe more research is necessary to resolve this uncertainty concerning pain experience in *Octopus vulgaris* adults?

Choose one of the following answers

☐ Yes
☐ No
☐ I do not believe there is uncertainty

Next

If the answer to the previous question was "yes", the question underneath will be shown. If any of the other answer options are chosen, the question underneath will not be shown, and the survey will automatically skip to the next question.

*What type(s) of research would you recommend to resolve this uncertainty?

Some explanation about the techniques:

- Behavioral studies:** Observing and analyzing how *Octopus vulgaris* behaves in different situations to understand its actions, learning, and problem-solving abilities.
- Molecular neurobiological analyses:** Examining the brain and nervous system of *Octopus vulgaris* at the molecular level, such as studying genes, proteins, and cells to understand how its nervous system works.
- Alternative methods:** Using lab-based techniques like studying octopus cells or tissues in a dish, or computer models, instead of experimenting on live animals.

Select all that apply

☐ Behavioral studies
☐ Molecular neurobiological analyses
☐ Alternative methods (e.g. in vitro studies excluding "real" animal models)
☐ Other:

Next

*On a scale from 1 to 5, how ethical do you find the use of *Octopus vulgaris* adults in the context of neurobiological experiments?

🔴 *Octopus vulgaris* is a key species in neurobiology research. The octopus can be lightly sedated by exposure to 1% MgCl₂ for 10 minutes, after which it can be fully anesthetized by exposure to 1% isoflurane for 5 minutes. Following this treatment, the animal can recover, with recovery taking up to 20 minutes. A lethal dose of MgCl₂ can be used to euthanize (= kill) the animal; 3.5% MgCl₂ usually stops breathing within 15 minutes, and after 30 minutes, the octopus cannot be revived.

1 = not ethical at all
5 = completely ethical

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate the societal relevance of neurobiological research on *Octopus vulgaris* adults?

🔴 Research is societally relevant when it answers questions that society asks, or solves problems that society faces.

1 = not relevant at all
5 = highly relevant

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*To what extent do you agree with the following statement: "The societal relevance of this research ethically outweighs the potential pain experienced by adult *O. vulgaris*:"?

🔴 1 = strongly disagree
5 = strongly agree

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate your English proficiency?

🔴 1 = no proficiency - you cannot understand or communicate in English at all
5 = native speaker level - you are highly proficient and communicate fluently and accurately in all situations

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

Thank you for completing the survey!

My name is Jill Monnissen. I am currently in my second, and last, year of the Master of Biology at KU Leuven. This research study is part of my master's thesis about sentience in *Octopus vulgaris* paralarvae. For my thesis I wanted to investigate what is known about pain experience in *O. vulgaris*, and how people's ethical judgements and beliefs about handling both adult and paralarval *O. vulgaris* might change according to their background and previously received information.

In this survey, you've read an abstract containing information about how researchers studied the expression of nociceptive genes, previously identified in the adults, in the paralarval stage of *Octopus vulgaris*. I am actually the one who performed this experiment as a part of my master's thesis as well. I was able to identify nine different nociceptive genes, previously identified in the adult, in the *O. vulgaris* paralarvae, suggesting the paralarvae are able to experience pain.

It might be the case you've received different information in the abstract. Either the abstract stated no nociceptive genes were found in the paralarval stage, or the experiment had not yet been conducted. However, this "incorrect" information was given to you for a reason: to see whether people's ethical judgements and beliefs are affected based on the information they receive before filling in the survey.

I would like to thank you one last time for cooperating on this research study and helping me with my master's thesis project! I hope everything is clear after this final disclaimer. If you still have some questions about this study and/or are interested in the results, feel free to contact me (jill.monnissen@student.kuleuven.be) or one of my supervisors (andreas.deblock@kuleuven.be or eve.seuntjens@kuleuven.be).

Kind regards,
Jill Monnissen
Master of Biology
KU Leuven

Non-Biologists

*To which group do you belong?

● Choose one of the following answers

- ☐ Biologist performing research using cephalopods (Octopus vulgaris and other species)
- ☐ Biologist performing research using other (model) organisms
- ☐ Non-biologist

Next

*How old are you?

● Choose one of the following answers

- ☐ 18 - 30 years old
- ☐ 31 - 40 years old
- ☐ 41 - 50 years old
- ☐ 50+ years old

Next

*What is your nationality?

● Choose one of the following answers

- ☐ American
- ☐ Belgian
- ☐ British
- ☐ Canadian
- ☐ Chinese
- ☐ Dutch
- ☐ French
- ☐ German
- ☐ Greek
- ☐ Indian
- ☐ Italian
- ☐ Japanese
- ☐ Mexican
- ☐ Nigerian
- ☐ Portuguese
- ☐ Russian
- ☐ Spanish
- ☐ Swedish
- ☐ Other:

Next

***How do you identify?**

☒ Choose one of the following answers

☐ Male

☐ Female

☐ Non-binary

☐ I prefer not to answer this question

☐ Other:

Next

***What is your highest level of education?**

☒ Choose one of the following answers

☐ High school graduate

☐ Bachelor's degree

☐ Master's degree

☐ Doctorate degree

☐ Other:

Next

***On a scale from 1 to 5, how familiar are you with animal research ethics?**

☒ 1 = not familiar at all

5 = very familiar

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

In this next “question,” one of three different abstracts will appear: Scenario 1, Scenario 2, or Scenario 3. The abstract that appears is completely random and will change each time the survey is started.

Scenario 1:

***Read the following abstract:**

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Recently, researchers studied the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris*. Analysis was conducted using state-of-the-art molecular biological techniques. They found evidence that these genes are expressed in the paralarval stage.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

Scenario 2:

*Read the following abstract:

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers at KU Leuven are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Researchers studied the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris* to get a better understanding of nociception in this stage. Analysis was conducted using state-of-the-art molecular biological techniques. They found no evidence of these genes being expressed in the paralarval stage.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

Scenario 3:

*Read the following abstract:

The paralarval stage of the common octopus, *Octopus vulgaris*, is an emerging model organism in neuroscience research. These paralarvae are free-living and self-feeding three days after hatching, exhibiting complex behaviors such as swimming, hunting, and camouflage. Researchers at KU Leuven are particularly interested in understanding the neurobiological mechanisms underlying these behaviors. Previous studies have identified several genes in adult *O. vulgaris* that are linked to nociception, the process by which potentially harmful stimuli are detected and processed. Researchers plan to study the expression of these nociceptive genes, previously identified in the adult organisms, in the paralarval *O. vulgaris* to get a better understanding of nociception in this stage. Analysis will be conducted using state-of-the-art molecular biological techniques. The experiment has not yet been conducted.

After reading the abstract, please confirm and answer the following questions.

☒ Choose one of the following answers

☐ Confirm you have read the abstract above

*Do you think paralarval *Octopus vulgaris* individuals experience pain?



Yes



No

Next

*On a scale from 1 to 5, how confident are you in your interpretation of whether paralarval *Octopus vulgaris* individuals might experience pain or not?

☒ 1 = very uncertain

5 = very certain

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you believe more research is necessary to resolve this uncertainty concerning pain experience in *Octopus vulgaris* paralarvae?

☒ Choose one of the following answers

☐ Yes

☐ No

☐ I do not believe there is uncertainty

Next

If the answer to the previous question was “yes”, the question underneath will be shown. If any of the other answer options are chosen, the question underneath will not be shown, and the survey will automatically skip to the next question.

*What type(s) of research would you recommend to resolve this uncertainty?

Some explanation about the techniques:

- **Behavioral studies:** Observing and analyzing how *Octopus vulgaris* behaves in different situations to understand its actions, learning, and problem-solving abilities.
- **Molecular neurobiological analyses:** Examining the brain and nervous system of *Octopus vulgaris* at the molecular level, such as studying genes, proteins, and cells to understand how its nervous system works.
- **Alternative methods:** Using lab-based techniques like studying octopus cells or tissues in a dish, or computer models, instead of experimenting on live animals.

Select all that apply

☐ Behavioral studies

☐ Molecular neurobiological analyses

☐ Alternative methods (e.g. in vitro studies excluding "real" animal models)

☐ Other:

Next

*On a scale from 1 to 5, how ethical do you find the use of *Octopus vulgaris* paralarvae in the context of neurobiological experiments?

The paralarvae are first sedated by exposing them to 2.5% ethanol and after they can be exposed to 4% ethanol to kill them. Dead paralarvae are used to make sections for studying gene expression.

1 = not ethical at all
5 = completely ethical

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate the societal relevance of neurobiological research on *Octopus vulgaris* paralarvae?

Research is societally relevant when it answers questions that society asks, or solves problems that society faces.

1 = not relevant at all
5 = highly relevant

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*To what extend do you agree with the following statement: "The societal relevance of this research ethically outweighs the potential pain experienced by paralarval *O. vulgaris*."

1 = strongly disagree
5 = strongly agree

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you think adult *Octopus vulgaris* individuals experience pain?

☒ Yes
 ☐ No

Next

*On a scale from 1 to 5, how confident are you in your interpretation of whether adult *Octopus vulgaris* individuals might experience pain or not?

1 = very uncertain
5 = very certain

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*Do you believe more research is necessary to resolve this uncertainty concerning pain experience in *Octopus vulgaris* adults?

Choose one of the following answers

☐ Yes
☐ No
☐ I do not believe there is uncertainty

Next

If the answer to the previous question was “yes”, the question underneath will be shown. If any of the other answer options are chosen, the question underneath will not be shown, and the survey will automatically skip to the next question.

*What type(s) of research would you recommend to resolve this uncertainty?

Some explanation about the techniques:

- Behavioral studies:** Observing and analyzing how *Octopus vulgaris* behaves in different situations to understand its actions, learning, and problem-solving abilities.
- Molecular neurobiological analyses:** Examining the brain and nervous system of *Octopus vulgaris* at the molecular level, such as studying genes, proteins, and cells to understand how its nervous system works.
- Alternative methods:** Using lab-based techniques like studying octopus cells or tissues in a dish, or computer models, instead of experimenting on live animals.

Select all that apply

☐ Behavioral studies
☐ Molecular neurobiological analyses
☐ Alternative methods (e.g. in vitro studies excluding “real” animal models)
☐ Other:

Next

*On a scale from 1 to 5, how ethical do you find the use of *Octopus vulgaris* adults in the context of neurobiological experiments?

① *Octopus vulgaris* is a key species in neurobiology research. The octopus can be lightly sedated by exposure to 1% MgCl₂ for 10 minutes, after which it can be fully anesthetized by exposure to 1% isoflurane for 5 minutes. Following this treatment, the animal can recover, with recovery taking up to 20 minutes. A lethal dose of MgCl₂ can be used to euthanize (= kill) the animal; 3.5% MgCl₂ usually stops breathing within 15 minutes, and after 30 minutes, the octopus cannot be revived.

1 = not ethical at all
5 = completely ethical

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate the societal relevance of neurobiological research on *Octopus vulgaris* adults?

① Research is societally relevant when it answers questions that society asks, or solves problems that society faces.

1 = not relevant at all
5 = highly relevant

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*To what extent do you agree with the following statement: "The societal relevance of this research ethically outweighs the potential pain experienced by adult *O. vulgaris*:"?

① 1 = strongly disagree
5 = strongly agree

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

*On a scale from 1 to 5, how would you rate your English proficiency?

① 1 = no proficiency - you cannot understand or communicate in English at all
5 = native speaker level - you are highly proficient and communicate fluently and accurately in all situations

	1	2	3	4	5
	☐	☐	☐	☐	☐

Next

Thank you for completing the survey!

My name is Jill Monnissen. I am currently in my second, and last, year of the Master of Biology at KU Leuven. This research study is part of my master's thesis about sentience in *Octopus vulgaris* paralarvae. For my thesis I wanted to investigate what is known about pain experience in *O. vulgaris*, and how people's ethical judgements and beliefs about handling both adult and paralarval *O. vulgaris* might change according to their background and previously received information.

In this survey, you've read an abstract containing information about how researchers studied the expression of nociceptive genes, previously identified in the adults, in the paralarval stage of *Octopus vulgaris*. I am actually the one who performed this experiment as a part of my master's thesis as well. I was able to identify nine different nociceptive genes, previously identified in the adult, in the *O. vulgaris* paralarvae, suggesting the paralarvae are able to experience pain.

It might be the case you've received different information in the abstract. Either the abstract stated no nociceptive genes were found in the paralarval stage, or the experiment had not yet been conducted. However, this "incorrect" information was given to you for a reason: to see whether people's ethical judgements and beliefs are affected based on the information they receive before filling in the survey.

I would like to thank you one last time for cooperating on this research study and helping me with my master's thesis project! I hope everything is clear after this final disclaimer. If you still have some questions about this study and/or are interested in the results, feel free to contact me (jill.monnissen@student.kuleuven.be) or one of my supervisors (andreas.deblock@kuleuven.be or eve.seuntjens@kuleuven.be).

Kind regards,
Jill Monnissen
Master of Biology
KU Leuven

8.1.2. Figures – Expression Patterns of Genes Related to Nociception

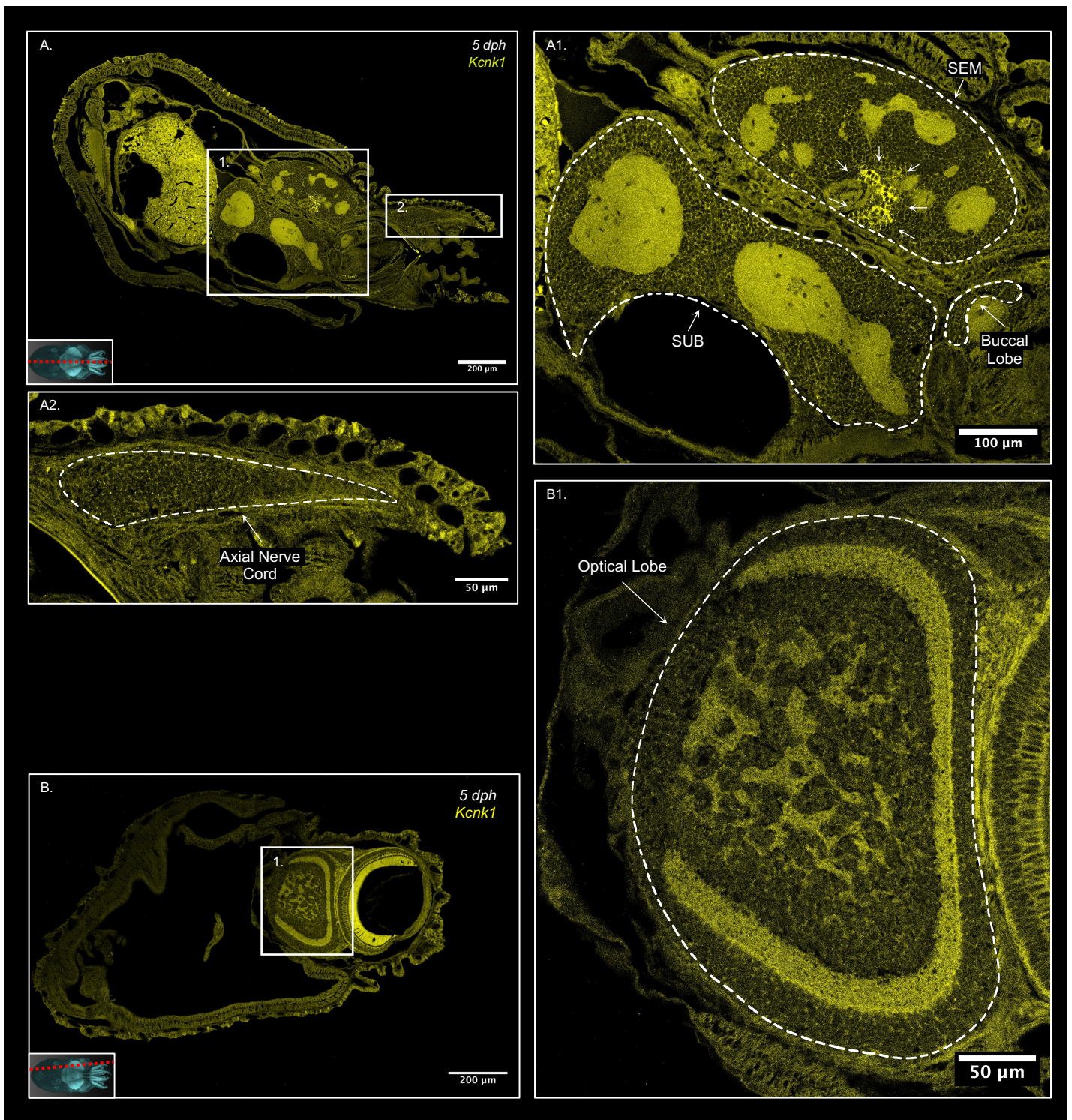


Figure A1 (continued on next page): Gene expression of *Kcnk1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Kcnk1* gene expression. White squares indicate regions shown at higher magnification in panels A1 and A2. The inset in the bottom left corner illustrates the cutting plane used to obtain the section. The scale bar represents 200 μm . (A1) Magnified view of the supraesophageal mass (SEM), subesophageal mass (SUB), and buccal lobe at 5 dph, showing strong *Kcnk1* expression. White arrows in the SEM highlight very strong *Kcnk1* expression in the basal lobe. The scale bar represents 100 μm . (A2) Magnified view of the axial nerve cord showing weak *Kcnk1* expression along its length at 5 dph. The scale bar represents 50 μm . (B) Sagittal section of another 5 dph *O. vulgaris* paralarva, cut along a different plane. The white square indicates the area enlarged in panel B1. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (B1) Magnified view of the optical lobe showing localized and quite weak *Kcnk1* expression at 5 dph. The scale bar represents 50 μm .

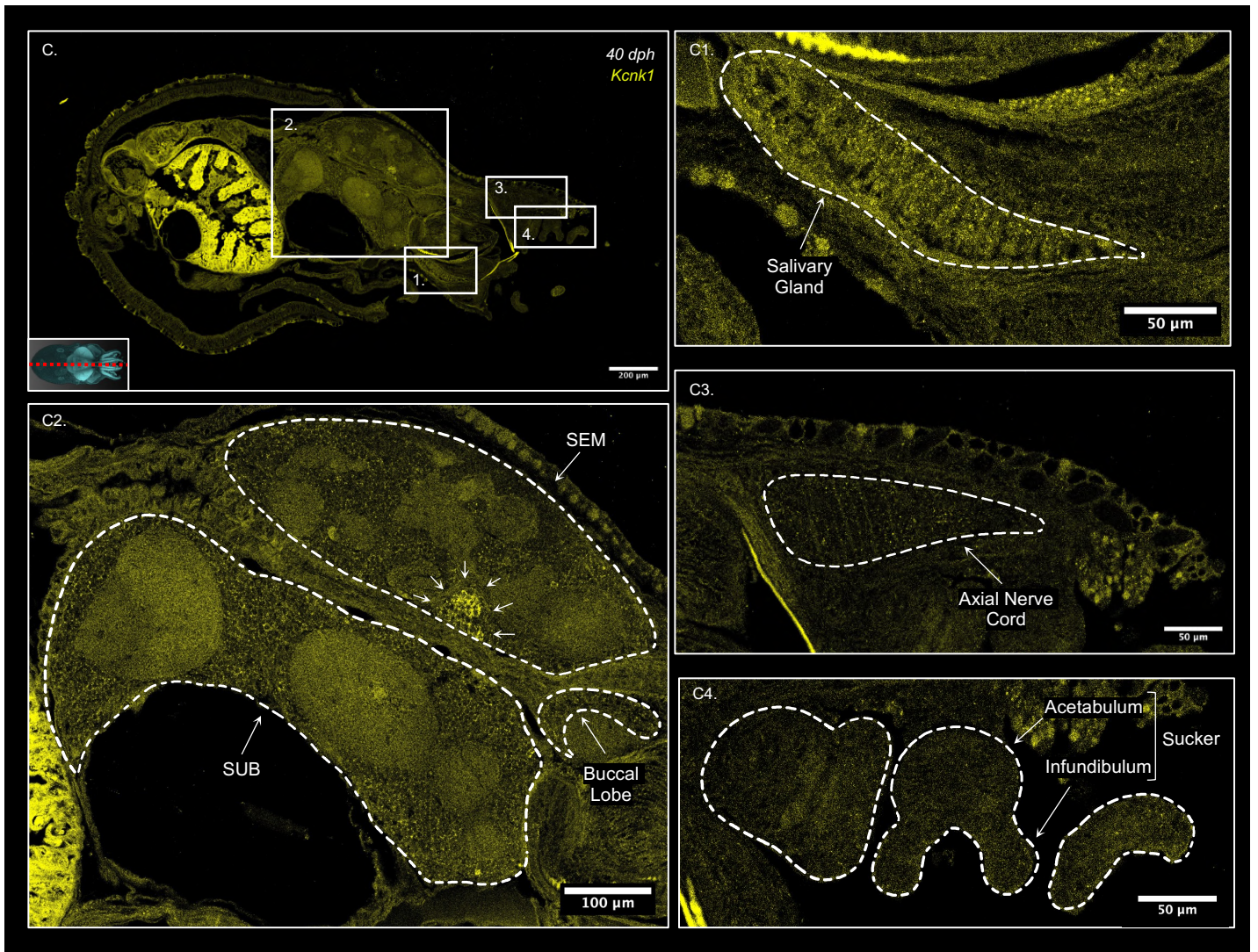


Figure A1 (continued): Gene expression of *Kcnk1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (C) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Kcnk1* gene expression. White squares indicate the regions shown at higher magnification in panels C1, C2, C3, and C4. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm in actual size. (C1) Magnified view of the salivary gland at 40 dph, showing strong *Kcnk1* expression. The scale bar represents 50 μm . (C2) Magnified view of the supraesophageal mass (SEM), subesophageal mass (SUB), and buccal lobe at 40 dph, showing very strong *Kcnk1* expression. The white arrows in the SEM highlight very strong *Kcnk1* expression in the basal lobe, consistent with the pattern observed at 5 dph. The scale bar represents 100 μm . (C3) Magnified view of the axial nerve cord at 40 dph, showing strong expression of *Kcnk1*. The scale bar represents 50 μm . (C4) Magnified view of the suckers at 40 dph, showing weak *Kcnk1* expression in the acetabulum and infundibulum. The scale bar represents 50 μm .

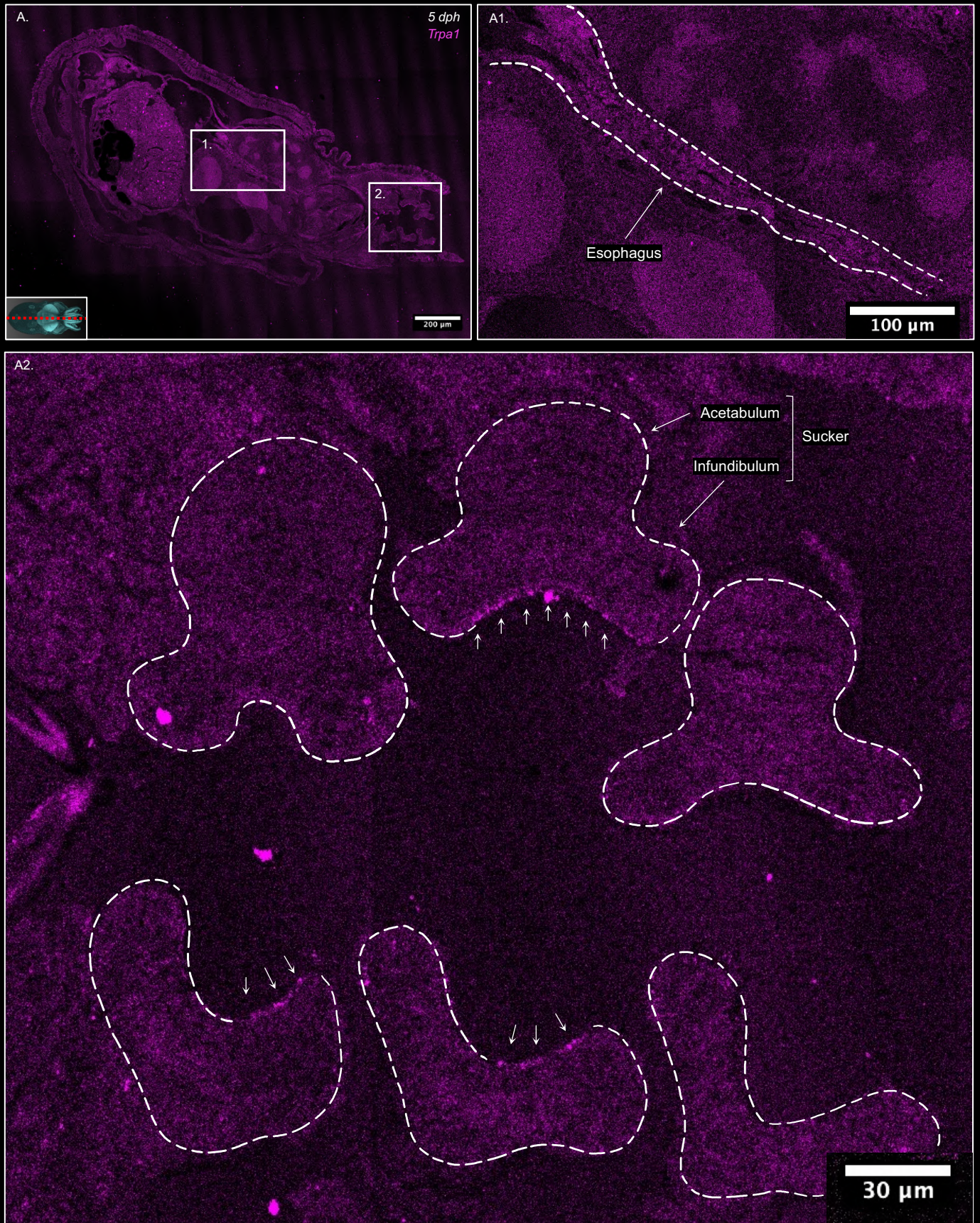


Figure A2 (continued on next page): Gene expression of *Trpa1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Trpa1* gene expression. The white squares indicate the regions shown at higher magnification in panels A1 and A2. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (A1) Magnified view of the esophagus at 5 dph, showing weak *Trpa1* expression. The scale bar represents 100 μm . (A2) Magnified view of the suckers at 5 dph, showing very strong *Trpa1* expression in the infundibulum. This expression is indicated by the white arrows. The scale bar represents 30 μm .

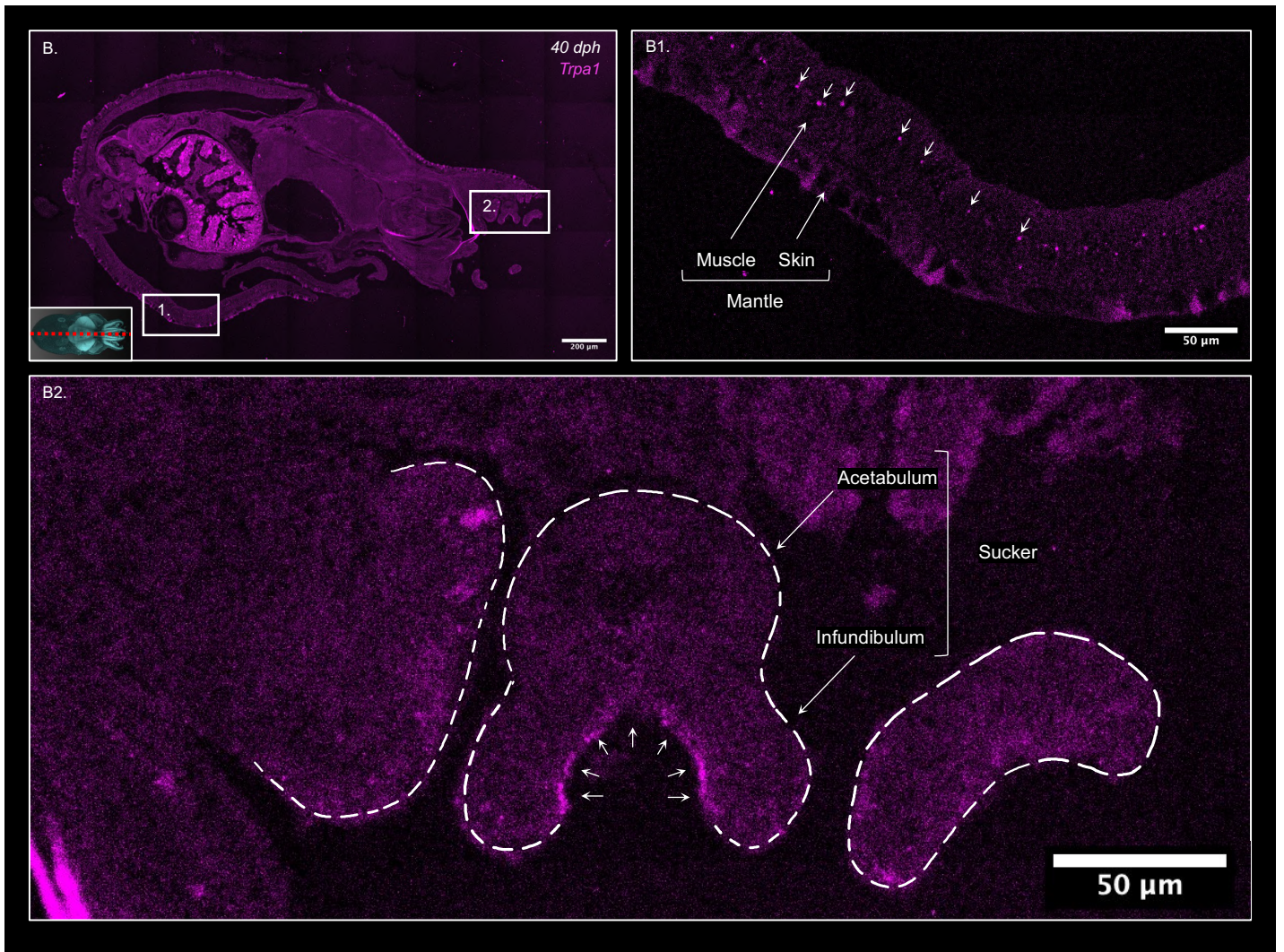


Figure A2 (continued): Gene expression of *Trpa1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (B) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Trpa1* gene expression. The white squares indicate the regions shown at higher magnification in panels B1 and B2. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (B1) Magnified view of the mantle at 40 dph. Strong *Trpa1* expression is visible in the muscle layer of the mantle, where it appears to follow a distinct linear pattern. The expression is indicated by white arrows. The scale bar represents 50 μm . (B2) Magnified view of the suckers at 40 dph, again showing very strong *Trpa1* expression in the infundibulum, as indicated by the white arrows. This expression pattern is consistent with that observed at 5 dph. The scale bar represents 50 μm .

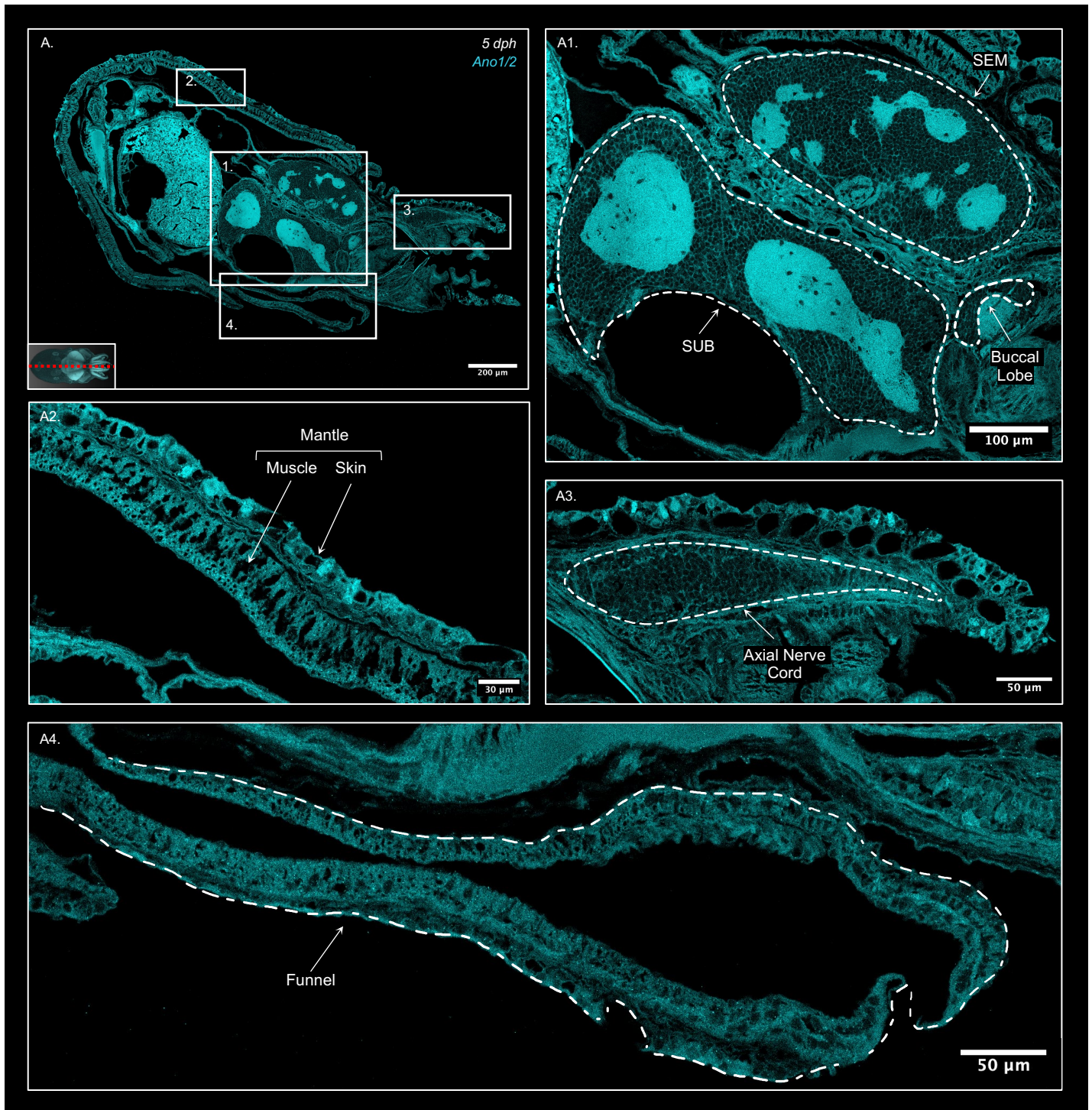


Figure A3 (continued on next page): Gene expression of *Ano1/2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Ano1/2* gene expression. The white squares indicate the regions shown at higher magnification in panels A1, A2, A3, and A4. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (A1) Magnified view of the supraesophageal mass (SEM) and subesophageal mass (SUB) at 5 dph, showing very weak *Ano1/2* expression. The scale bar represents 100 μm . (A2) Magnified view of the mantle at 5 dph, showing strong *Ano1/2* expression, especially in the skin layer. The scale bar represents 30 μm . (A3) Magnified view of the axial nerve cord at 5 dph, showing weak *Ano1/2* expression along its length. The scale bar represents 50 μm . (A4) Magnified view of the funnel at 5 dph, showing strong *Ano1/2* expression. The scale bar represents 50 μm .

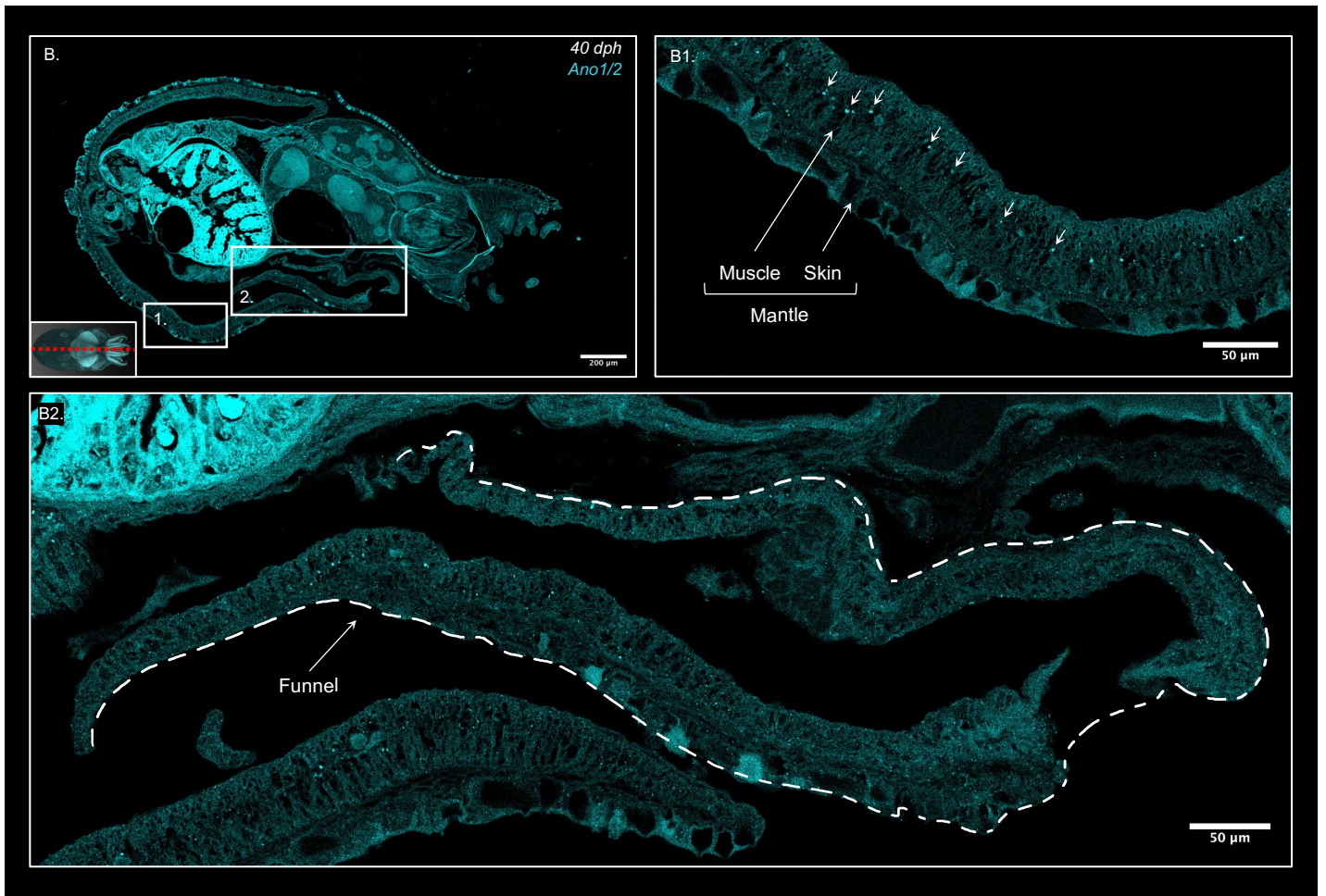


Figure A3 (continued): Gene expression of *Ano1/2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (B) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Ano1/2* gene expression. The white squares indicate the regions shown at higher magnification in panels B1 and B2. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 µm. (B1) Magnified view of the mantle at 40 dph, showing strong *Ano1/2* expression, particularly in the muscle layer. The expression pattern, which is highlighted by white arrows, follows a distinct linear pattern. The scale bar represents 50 µm. (B2) Magnified view of the funnel at 40 dph, showing strong *Ano1/2* expression along the epithelial tissue. The scale bar represents 50 µm.

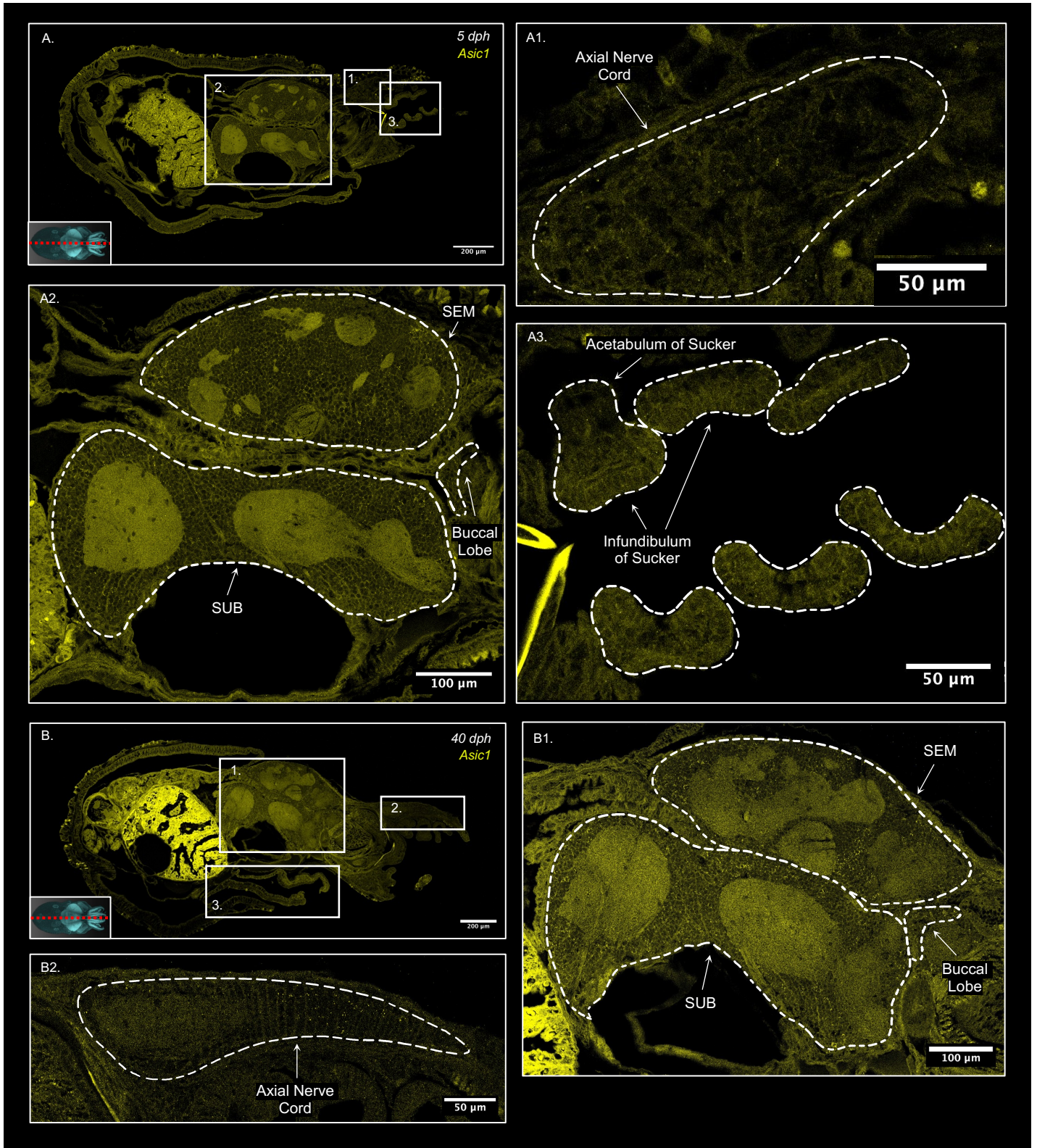


Figure A4 (continued on next page): Gene expression of *Asic1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Asic1* gene expression. The white squares indicate the regions shown at higher magnification in panels A1, A2, and A3. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μ m. (A1) Magnified view of the axial nerve cord at 5 dph showing very weak *Asic1* expression. The scale bar represents 50 μ m. (A2) Magnified view of the supraesophageal mass (SEM) and subesophageal mass (SUB) at 5 dph showing weak *Asic1* expression. The scale bar represents 100 μ m. (A3) Magnified view of the suckers at 5 dph, showing weak *Asic1* expression in both the acetabulum and infundibulum. The scale bar represents 50 μ m. (B) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Asic1* gene expression. The white squares indicate the regions shown at higher magnification in panels B1, B2, and B3 (next page). The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μ m. (B1) Magnified view of the SEM, SUB, and buccal lobe at 40 dph, showing strong expression of *Asic1*. The scale bar represents 100 μ m. (B2) Magnified view of the axial nerve cord at 40 dph, showing weak *Asic1* expression along its length. The scale bar represents 50 μ m.

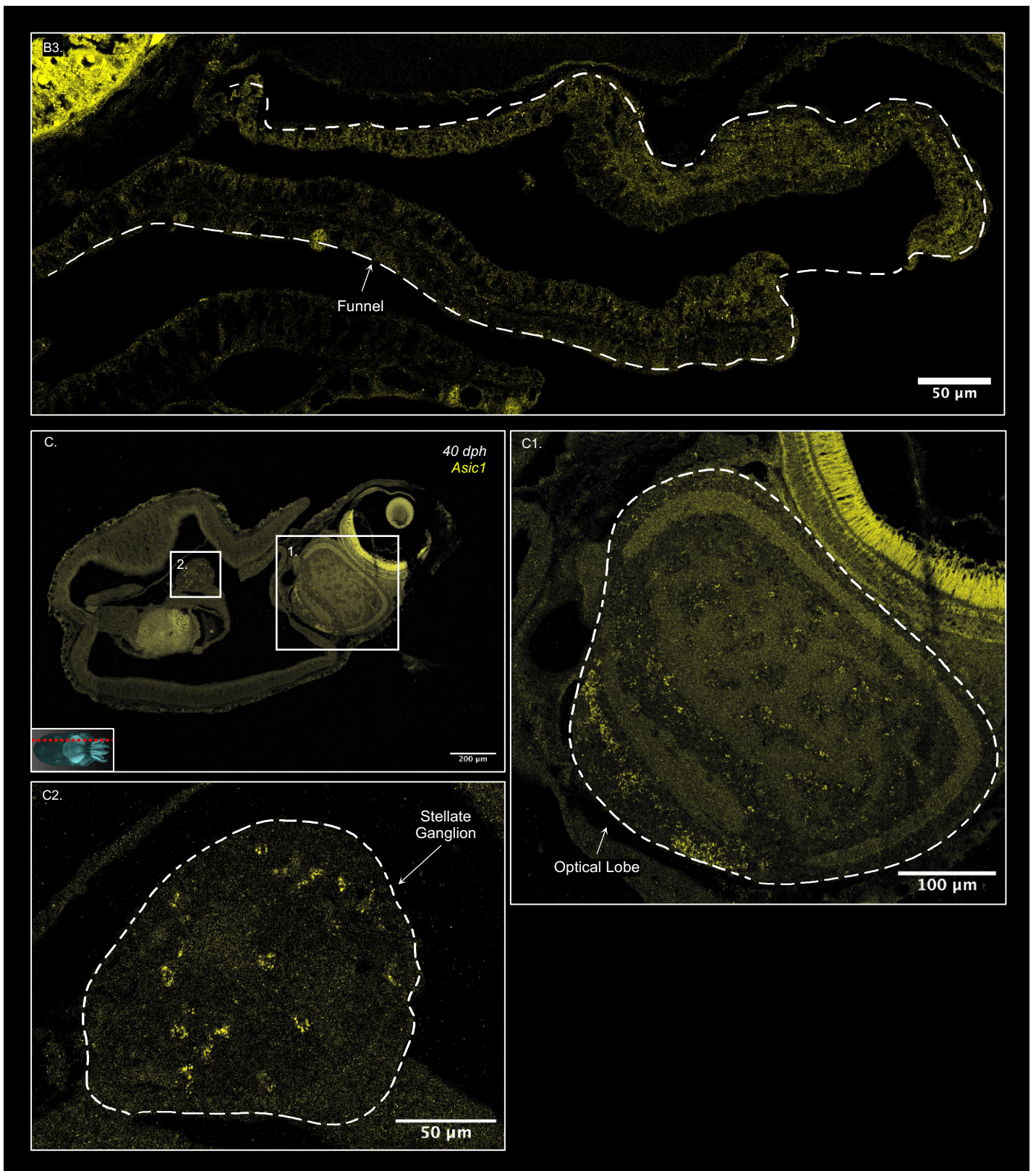


Figure A4 (continued): Gene expression of *Asic1* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (B3) Magnified view of the funnel at 40 dph, showing strong *Asic1* gene expression. This panel corresponds to a region shown in the overview panel B on the previous page. The scale bar represents 50 μm . (C) Sagittal section of a 40 dph *O. vulgaris* paralarva obtained at a different cutting plane, showing the expression of *Asic1*. The white squares indicate the regions shown at higher magnification in panels C1 and C2. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (C1) Magnified view of the optic lobe at 40 dph, showing very strong *Asic1* expression. The scale bar represents 100 μm . (C2) Magnified view of the stellate ganglion at 40 dph, also showing very strong expression of *Asic1*. The scale bar represents 50 μm .

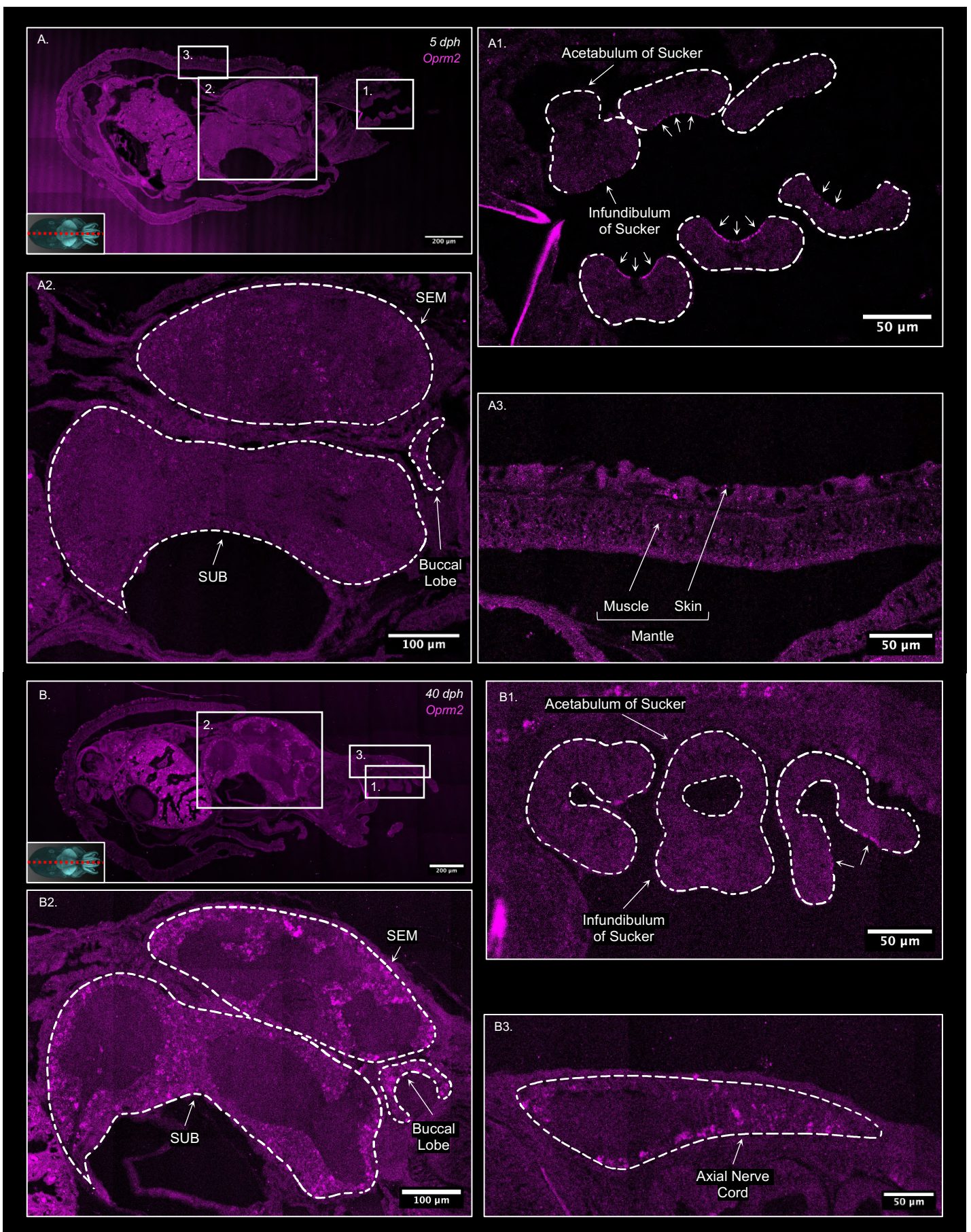


Figure A5 (continued on next page): Gene expression of *Oprm2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Oprm2* gene expression. The white squares indicate the regions shown at higher magnification in panels A1, A2, and A3. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μ m. (A1) Magnified view of the suckers at 5 dph, showing very strong expression of *Oprm2* in the infundibulum, indicated by the white arrows. The scale bar represents 50 μ m. (A2) Magnified view of the supraesophageal mass (SEM), subesophageal mass (SUB), and buccal lobe at 5 dph, showing strong *Oprm2* expression. The scale bar represents 100 μ m. (A3) Magnified view of the mantle at 5 dph, showing weak *Oprm2* expression in both the muscle and skin layers. The scale bar represents 50 μ m. (B) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Oprm2* gene expression. The white squares indicate the regions shown at higher magnification in panels B1, B2, and B3. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μ m. (B1) Magnified view of the suckers at 40 dph, again showing strong expression of *Oprm2* in the infundibulum, as indicated by the white arrows. The scale bar represents 50 μ m. (B2) Magnified view of the SEM, SUB, and buccal lobe at 40 dph, all showing very strong *Oprm2* expression. The scale bar represents 100 μ m. (B3) Magnified view of the axial nerve cord at 40 dph, showing strong *Oprm2* expression. The scale bar represents 50 μ m.

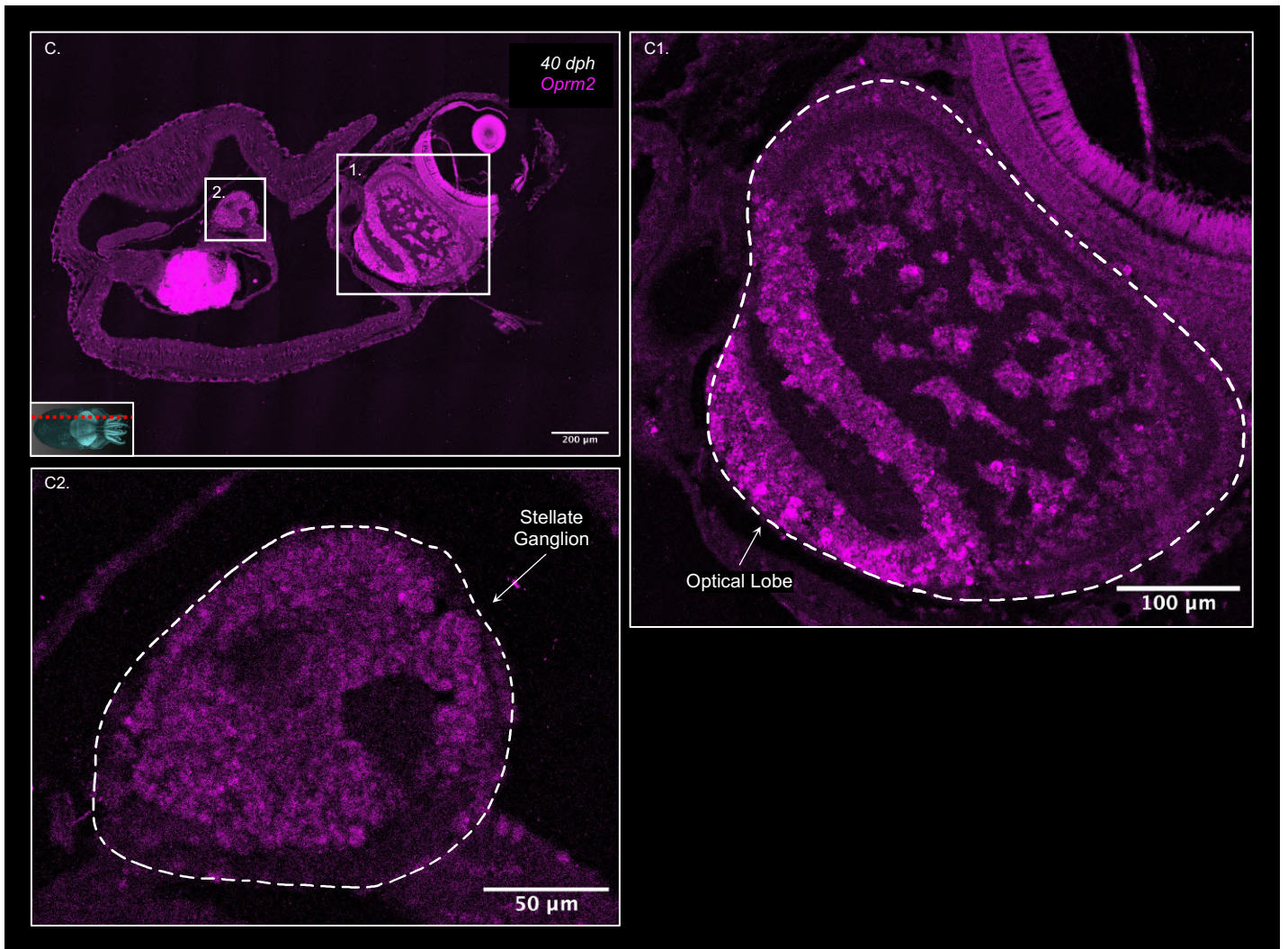


Figure A5 (continued): Gene expression of *Oprm2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (C) Sagittal section of a 40 dph *O. vulgaris* paralarva obtained at an alternative cutting plane, showing *Oprm2* expression. The white squares indicate the regions shown at higher magnification in panels C1 and C2. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μm . (C1) Magnified view of the optic lobe at 40 dph, showing very strong expression of *Oprm2*. The scale bar represents 100 μm . (C2) Magnified view of the stellate ganglion at 40 dph, also showing very strong expression of *Oprm2*. The scale bar represents 50 μm .

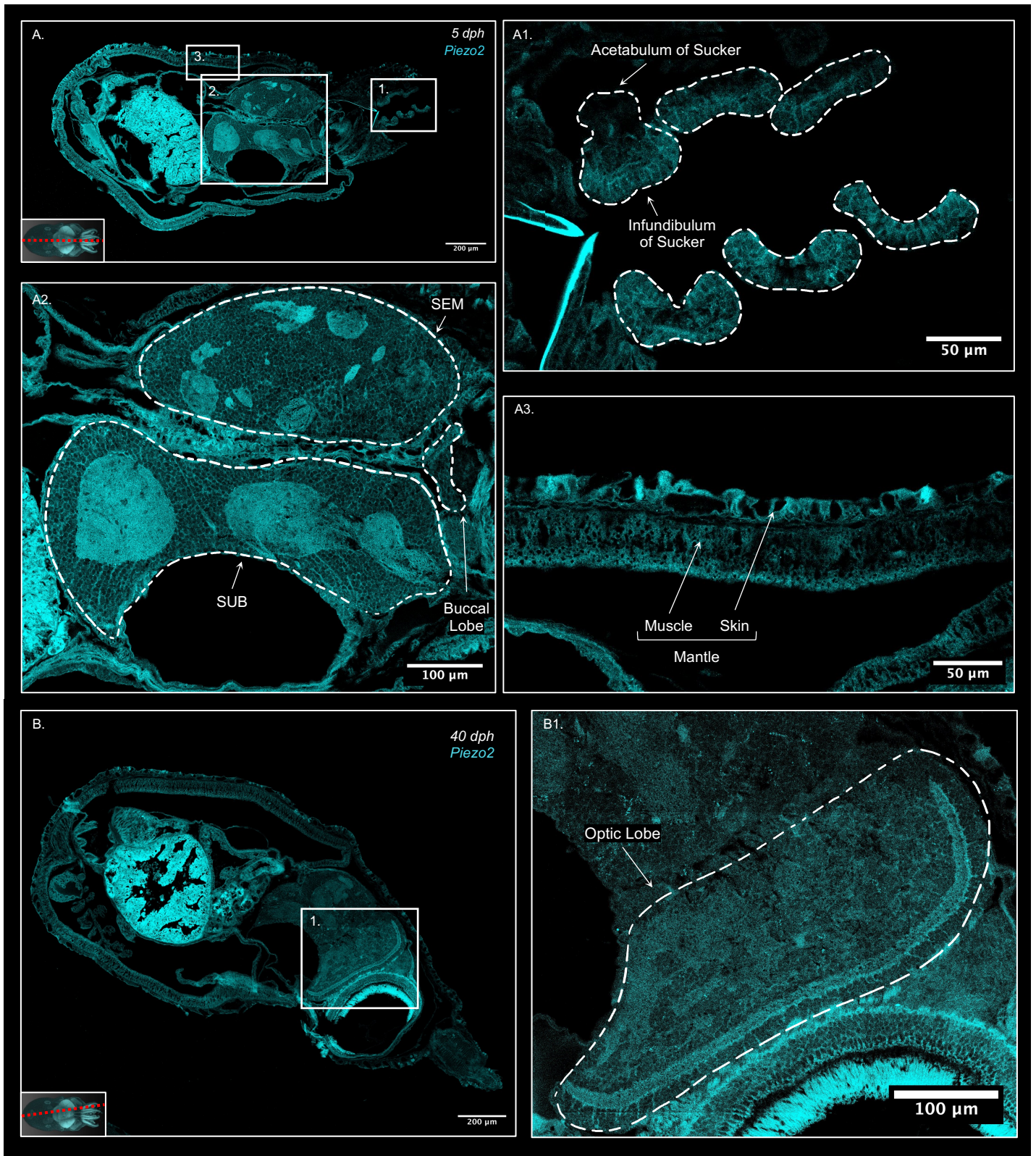


Figure A6 (continued on next page): Gene expression of *Piezo2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (A) Sagittal section of a 5 dph *O. vulgaris* paralarva showing *Piezo2* gene expression. The white squares indicate the regions shown at higher magnification in panels A1, A2, and A3. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 μ m. (A1) Magnified view of the suckers at 5 dph, showing weak *Piezo2* expression in both the acetabulum and infundibulum. The scale bar represents 50 μ m. (A2) Magnified view of the supraesophageal mass (SEM), subesophageal mass (SUB), and paliovisceral lobe at 5 dph, showing weak *Piezo2* expression. The scale bar represents 100 μ m. (A3) Magnified view of the mantle at 5 dph, showing weak *Piezo2* expression in both the muscle and skin layers. The scale bar represents 50 μ m. (B) Sagittal section of a 40 dph *O. vulgaris* paralarva showing *Piezo2* gene expression. The white square indicates the region shown at higher magnification in panel B1. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. As shown in the inset, the cutting plane is slightly tilted, resulting in some structures appearing distorted. The scale bar represents 200 μ m. (B1) Magnified view of the optic lobe at 40 dph, showing very weak *Piezo2* expression. Due to the tilted cutting plane, only part of the optic lobe is visible. The scale bar represents 100 μ m.

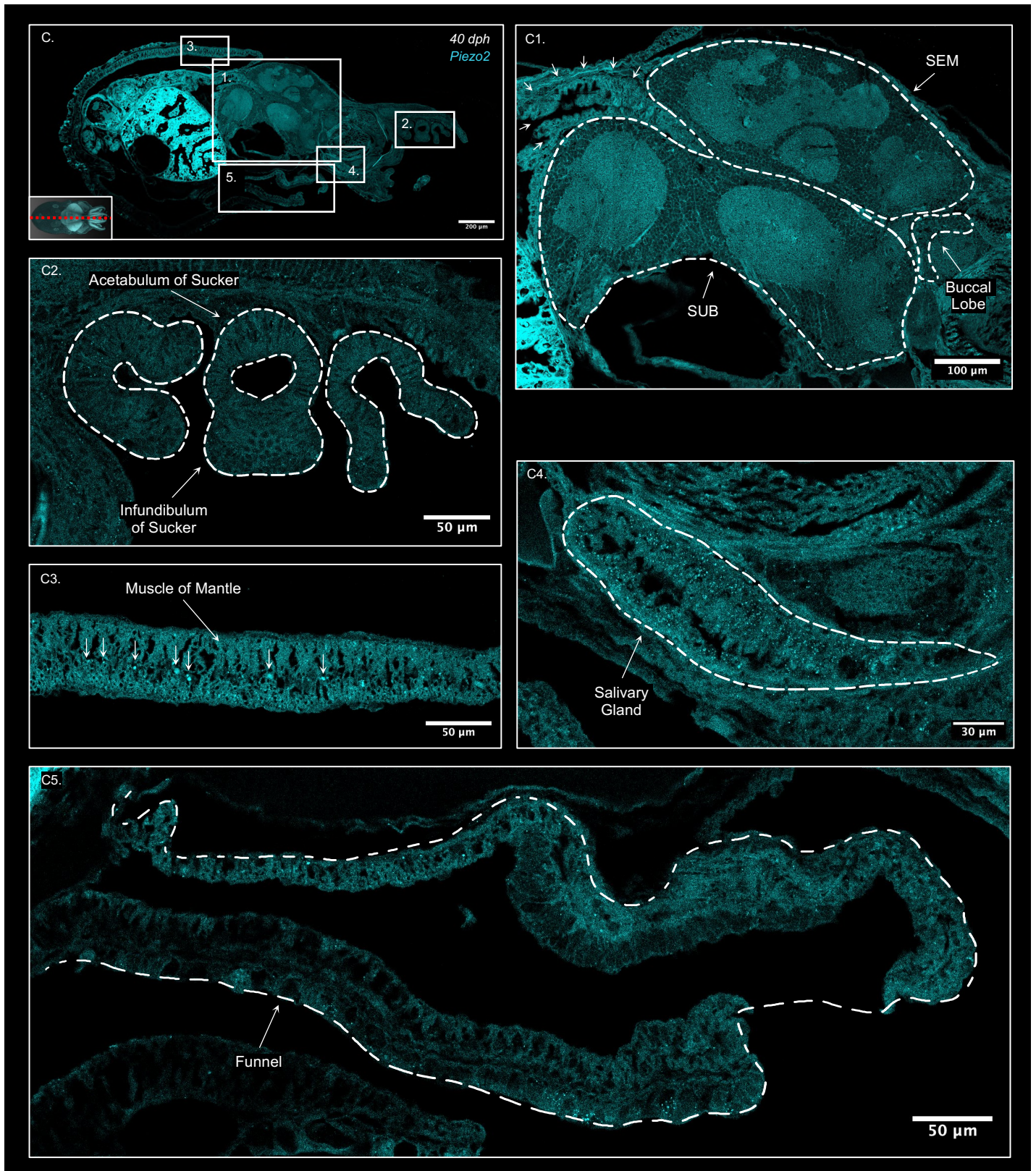


Figure A6 (continued): Gene expression of *Piezo2* in *Octopus vulgaris* paralarvae at 5 and 40 days post-hatching (dph). (C) Sagittal section of a 40 dph *O. vulgaris* paralarva, obtained at a different cutting plane, showing *Piezo2* gene expression. The white squares indicate the regions shown at higher magnification in panels C1, C2, C3, C4, and C5. The inset in the bottom left corner illustrates the cutting plane used to obtain this section. The scale bar represents 200 µm. (C1) Magnified view of the supraesophageal mass (SEM), subesophageal mass (SUB) and paliovisceral lobe at 40 dph, showing strong *Piezo2* expression. The white arrows indicate additional strong *Piezo2* expression observed in the esophagus (located between the SEM and SUB) and the surrounding gastrointestinal tract. The scale bar represents 100 µm. (C2) Magnified view of the suckers at 40 dph, showing weak *Piezo2* expression in both the acetabulum and the infundibulum. The scale bar represents 50 µm. (C3) Magnified view of the mantle at 40 dph, showing particularly strong *Piezo2* expression in the muscle layer. This expression follows a linear pattern and is indicated by white arrows. The scale bar represents 50 µm. (C4) Magnified view of the salivary gland at 40 dph, showing very strong *Piezo2* expression. The scale bar represents 30 µm. (C5) Magnified view of the funnel at 40 dph, showing strong *Piezo2* expression, particularly at the end of the funnel. The scale bar represents 50 µm.

8.2. Statement of AI Usage

In the preparation of this thesis, ChatGPT by OpenAI was used as a supportive tool to enhance the clarity and quality of the writing. Specifically, it assisted in structuring sentences and paragraphs, improving logical flow between sections, rephrasing when needed, and correcting grammar and language use. ChatGPT was also used to check that all references were consistently formatted according to APA 7th edition guidelines, including thesis-specific conventions (e.g., the use of *et al.* for works with more than ten authors). In addition, ChatGPT supported the process of statistical analysis. While I performed and interpreted all statistical tests myself, I consulted ChatGPT to identify suitable code, resolve recurring syntax or logic errors, and clarify output when needed. In cases of uncertainty, the tool was used to ensure correct interpretation of results. At no point was ChatGPT used to generate content independently or to write entire sections of the thesis. All substantive writing, interpretations, and conclusions are my own, and all information included in the thesis was verified against academic literature. The tool was used solely for assistance.

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