Title: Behavioral and physiological metrics of sensory decline and welfare in senescent giant Pacific octopus, *Enteroctopus dofleini*

Behavioral changes in senescent giant Pacific octopus (*Enteroctopus dofleini*) are associated with peripheral neural degeneration and loss of epithelial tissue.

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Abstract:

Most species of octopus experience extreme physical decline after a single reproductive bout which extends over a period of days, weeks, or months before eventual death. Although outward indicators of senescence are widely recognized, comparatively little is known about physiological and neural changes accompanying terminal decline in octopuses. Here, we measured changes in behavioral response to nociceptive stimuli across the lifespan in giant Pacific octopus (GPO), *Enteroctopus dofleini,* held in public aquariums in the USA. Post-euthanasia, tissue was collected from arm tips, and neural and epithelial cell degeneration was quantified and compared with biopsies of arm tips from healthy, pre-reproductive GPOs. Behavioral assays showed significant changes both in low threshold mechanosensory responses and nociceptive behavioral responses beginning early in senescence and extending until euthanasia. Histology data showed that while the ratio of apoptotic cells to total cell number stayed constant between healthy and senescent GPOs, overall neural and epithelial cell density was significantly lower in terminally senescent octopuses compared with healthy controls. Our data provide new insight into the time-course and causes of sensory dysfunction in senescent cephalopods and suggest proactive welfare management should begin early in the senescence phase, well before animals enter terminal decline.

Introduction

Interest in cephalopod models in research is growing (Fiorito et al., 2015) and cephalopods continue to be popular display animals in aquariums, inspiring visitors with their unique anatomy, color changing capabilities and intelligence (Seeley et al., 2016). Most cephalopods are semelparous, meaning they experience a single, terminal reproductive event followed by a period of senescence (Rocha, Guerra, & Gonzalez, 2001), which is characterized by various behavioral and physiological changes including anorexia, major deterioration of the skin and muscle, sinking of the eyes, stereotypic repetitive behaviors and, occasionally, self-mutilation and autophagy (Anderson et al., 2002; Holst & Miller-Morgan, 2020; Roumbedakis et al., 2018; Z. Wang, 2018; Z. Y. Wang & Ragsdale, 2018)

In the wild, male octopus may mate with more than one female before dying, while females will brood their eggs and die, typically by predation, shortly after eggs hatch (Rocha et al., 2001; Rosa, Costa, & Nunes, 2004). Changes in photoperiod cause the optic gland in females to secrete reproductive hormones, inducing egg laying, senescence, and rapid physical decline (Wodinsky, 2019). In captive environments, octopuses may not have the opportunity to mate, and are typically hand fed and receive end-of-life veterinary care. Therefore, senescence may be extended longer than what may be typical in the wild, raising questions about the ethics of maintaining senescent animals in research and public aquaria (Mather & Anderson, 2007).

Invertebrates are generally not protected by research animal welfare laws at the same level as vertebrates, but cephalopods are of increasing ethical concern for animal welfare governing bodies, as well as in zoos and aquariums (Birch et al., 2021; Browning, n.d.; Browning & Birch, 2022; Carere & Mather, 2019; Fiorito et al., 2015; Harvey-Clark, 2011; Holst & Miller-Morgan, 2020; Jacquet, Franks, Godfrey-Smith, et al., 2019; Mather, 2022; Moltschaniwskyj et al., 2007). It is currently unknown whether senescence in itself is a welfare concern, or how senescence-induced changes to octopus physiology may affect behavior and influence research findings. Certainly, skin lesions and other physical symptoms such as extreme weight loss, blindness, and autophagy, which are often observed in senescent animals, would be considered strong indicators of poor welfare in non-senescent animals. While aquarists' and researchers' tolerance of physical injuries may be higher for senescent octopuses, empirical studies of cephalopods' ability to experience suffering and distress as a result of tissue damage and other senescence-induced changes are still in their infancy (Crook, 2021). However, nociceptive plasticity resembling both allodynia and hyperalgesia has been documented in non-senescent cephalopods after tissue injury (Crook & Walters, 2011; Illich & Walters, 1997; Walters, 1987, 1994) and tissue damage produces a range of behavioral responses which are analogous to pain-related behavior in vertebrate animals. Senescent cephalopods do not appear to reliably produce the same kinds of responses to tissue damage as would be expected for healthy animals (Holst and Miller-Morgan, 2020), but whether this is due to sensory system degeneration, loss of motor control, or some other factor is completely unknown.

Here, we examine behavior of captive giant Pacific octopus, *Enteroctopus dofleini*, while they are healthy and follow them as they decline through senescence. First, weekly tests of mechanosensory and mechano-nociceptive thresholds were performed with von Frey filaments, allowing us to track within-individual changes in responsiveness over the lifespan. All animals in the study were eventually euthanized by their care team in late senescence, and tissue samples were collected from the arms for evaluation of degeneration in the neural and epithelial tissues of the arms. We show that changes to sensory processing begin earlier than previously reported, at the onset of reproductive behavior, and that declines in behavioral responsiveness to both noxious (harmful or potentially harmful) and non-noxious touch in the terminal phase are correlated with dramatic declines in overall cell density in the arm nerve cord and epithelial tissues of the arms.

Materials and Methods

Animals and participating institutions

Giant Pacific octopuses, *Enteroctopus dofleini*, are large animals capable of reaching over 120lbs (Cosgrove, 1976). Holding space for such large animals at any one institution is limited, posing challenges for studies requiring more than one or two replicates. Therefore, the host institution (Aquarium of the Bay, San Francisco, CA) sought outside participating public aquariums to replicate the procedures and increase sample size. A total of three additional institutions were recruited to replicate the host institution's protocols for sensory threshold tests for their resident *Enteroctopus dofleini* from initial enrollment in the study through senescence. A full list of each participating institution and the number of animals at each location is given in Table 1. Octopuses were held in off-display holding enclosures or public display enclosures under controlled conditions for diet, water quality and husbandry routines. Biologists performing sensory threshold testing and tissue harvest procedures were the primary staff biologist onsite at each respective location and remained consistent throughout the study. Outside institutions were trained through video sessions with the lead author (M.H.) to ensure consistent replication of procedures between all sites. Participating facilities were mailed identical sets of von Frey filaments (touch test sensory probes, Stoelting, Illinois, USA). Raw data was uploaded on a regular basis via Google Sheets that were shared between the host and the participating facilities for immediate review.

For each octopus in the study, the status of healthy (i.e., pre-reproductive) or senescent was determined through a standardized health assessment (Holst & Miller-Morgan, 2020), developed by the primary biologist responsible for *E. dofleini* management at Aquarium of the Bay in San Francisco (M.H). Senescent stages were determined by local caretakers in consultation with the first author (M.H.) (see Fig. 1 for examples of different senescence indicators). Initial, "low-concern" or Level 2 observations that become persistent were considered "early-senescence". Consistent "mid-concern" or Level 3 observations were considered "mid/late senescence", with animals reaching "high-concern" or Level 4 observations as "perimortem senescence" (see Holst & Miller-Morgan, 2020, for detailed descriptions of behavioral and physiological indicators of health).

Because recruitment occurred on an ongoing basis and the number of GPO specimens in public aquaria is limited and unpredictable, some animals were already within early, mid/late, or perimortem senescence when touch tests sequences began. Ultimately there were animals represented at each life stage of interest (pre-senescence $n = 3$, early senescence $n = 5$, mid/late senescence $n = 6$, and perimortem $n = 5$).

Sensory threshold testing

Mechanosensory and nociceptive thresholds for *E. dofleini* were quantified via touch-tests, using 0.16g, 1g, 10g, 26g and 60g von Frey filaments (Stoelting, Chicago, IL, USA). These filaments were chosen based on pilot observations of a single, healthy *E. dofleini*, and represent a range of stimulus intensities from minimally detectable to likely noxious. Animals were tested for response at the distal and proximal portion of one arm, and on the mantle between the eyes. Behavioral responses to application of each filament were ranked from 0-7 (Table 2). Touch test location occurred in consistent order for each filament, starting with the mantle, base-of-arm, and ending at the tip-of-arm location for each filament. Intervals between successive touches were judged by the testers discretion and were typically driven by the behavior of the animal and accessibility to each test location. Testers performed complete touch tests at roughly the same time of day and typically occurred once every week unless the animal became inaccessible part way through the test. Completing a touch test weekly was not always possible, particularly during the COVID-19 pandemic, as tester availability became unpredictable.

Tissue sample collection for histological analysis

Samples of arm tips were acquired from healthy (n = 2) and postmortem (n = 9) *E. dofleini*. Healthy arm tissue samples were live animal biopsies taken from two sub-adult octopuses at the primary institution and were acquired by removing roughly 3 mm of tissue from the distal end of one arm using a sterile scalpel. Prior to arm tip removal, the arm nerve cord was injected with between 1-2ml of aqueous magnesium chloride (MgCl2; 75g/L stock solution) proximal to the biopsy site to block sensation at the arm tip. There was no observed bleeding of the biopsy site, and the arm tip appeared to be completely healed upon examination in the weeks after.

All samples from terminally senescent animals (n=9) were acquired immediately (within 10-15 minutes of cessation of respiration, which typically precedes complete cessation of neural activity in the CNS by about 10 minutes) upon euthanasia at their home institution. Euthanasia was achieved via immersion in magnesium chloride for all specimens, at a concentration of 75g/L dissolved in Reverse Osmosis (RO) water (Messenger et al. 1985). Participating facilities then harvested approximately 1-inch-long arm sections from the distal ends of senescent *E. dofleini* arms.

Both healthy and senescent biopsies were fixed in 4% paraformaldehyde in seawater for 24 hours, then washed and stored in filtered, sterile seawater. Samples from institutions outside of San Francisco were shipped on ice priority overnight to the Crook Laboratory for histological analysis.

Tissue preparation and staining

Prior to sectioning, tissue was cryoprotected overnight in 30% sucrose solution until tissue pieces sank to the bottom of the vial. Samples were sectioned in the longitudinal plane, at 20µm on a cryostat (Leica). Sections were mounted on glass slides (Superfrost Plus, Fisherbrand, United

States), and kept at -20 degrees C prior to staining. Sections chosen for labeling included the axial nerve cord, at least one sucker, and dorsal epithelium (Figure 2), allowing for comparisons between all tissue types within a sample as well as across samples from different animals.

Tissue was first stained with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) using the In Situ Cell Death Detection (Flourescein) Kit (Roche, Basel, Switzerland, item 11684795910 from MilliporeSigma, USA). We followed the manufacturer's protocol for all labeling experiments. After TUNEL staining was complete, nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) at 1:500 dilution for ten minutes. Negative controls (TUNEL label without enzyme) were also labeled with DAPI. Labeled sections were mounted in Vectashield mounting medium and cover-slipped for imaging.

Tissue imaging and analysis

Samples were imaged in 8-layer z-stacks using a confocal microscope (Zeiss LSM 710) at 20x, concentrating on three locations of interest (epithelial tissue, axial (arm) ganglion, and sucker edge). Images were stacked and a maximum projection was made for each stack for analysis using FIJI. TUNEL and DAPI labeling were analyzed in ImageJ (2.1.0/1.53c) counting all TUNEL and DAPI positive cells in three randomly selected 5.71μm^2 areas, for each of the three anatomical regions of the arm we hypothesized would be most likely to reveal sensory receptor and neuronal decline.

Data analysis and statistical approaches

All data were analyzed in Prism (version 9.3.1). The critical alpha for each test was set at 0.05, and all reported p-values are two-tailed and corrected for multiple comparisons.

Behavioral data: Response thresholds (Table 2) were recorded for each filament at each test location (mantle, arm base, and arm tip) and responses were compared among different senescence stages. Kruskal-Wallis tests for non-parametric data were used for overall comparisons, and where significant effects were found, were followed by post-hoc, Dunn's tests for multiple comparisons. Because not all animals were tested at each stage of senescence, we treated responses at each stage of senescence as independent samples.

Histological data: We selected one section at random from each biopsy to perform TUNEL and DAPI staining, and one section was stained as a negative control (no TUNEL but with DAPI). Within each section there were three distinct anatomical areas on which we focused; the edge of the sucker (representing a densely innervated sensory epithelium), the cortical layer of an axial ganglion (containing the neuronal cell bodies associated with the sucker and local arm region), and the dorsal epithelium of the arm (comprising 'typical' octopus skin with chromatophores and various sensory structures, and is where skin degeneration and ulceration is often noted during terminal senescence, and is also the surface on which sensory threshold tests were conducted). Within each anatomical region, (sucker edge, ganglion and dorsal epithelium), results from three replicate, non-overlapping ROIs were averaged for statistical analysis. Because biopsies from healthy GPOs are exceptionally difficult to obtain, we used two sections from each of the healthy animals ($n = 2$) as technical replicates (i.e., four total sections from healthy, pre-reproductive animals).

For histological samples, the count of TUNEL+ cells was divided by the number of DAPI+ cells, to determine the proportion of dying cells within each sample. Proportions were calculated for each of the three ROIs per anatomical area (sucker margin, ganglion and dorsal epithelium), and then these values were averaged to produce a single mean value for each location for each animal. Unpaired t-tests were used to compare TUNEL/DAPI ratios between healthy and senescent animals. Overall numbers of DAPI+ cells were also compared for each tissue type, using unpaired t-test between healthy and senescent animals, to determine if overall cell density changed through senescence. To determine whether degree of tissue decline was reliably predicted by outward animal health scores, we used a linear regression of cell density counts from each region per animal against the number of "high concern" behavioral indicator scores (Holst & Miller-Morgan, 2020) that animal received in evaluations immediately prior to euthanasia.

Results

Touch-test behavioral analysis

While a series of 5 von Frey filaments (0.16g, 1g, 10g, 26g, 60g) were tested for each animal throughout the study, we chose only the lightest and heaviest filaments (0.16g and 26g) as the focus of detailed analysis, as they were the filaments with the most consistent successful applications in touch test procedures that were conducted by on-site caretakers. These filaments represent a clearly sub-nociceptive stimulus (0.16g) and a likely nociceptive stimulus (26g).

For the non-noxious 0.16g von Frey filament, there was a significant difference in response rank at the arm tip location over time (Kruskall-Wallis test, KW=8.33, p=0.026). Post-hoc Dunn's tests showed a significantly greater behavioral response to touch on the arm tip between pre- and early senescence (Dunn's test, $p = 0.0396$; Fig. 4A). Although there was a trend of increasing responses to the 0.16g filament at both the arm base and the mantle test location the differences were not significant (Figure 3).

For the potentially noxious 26g von Frey filament, there was a significant difference in behavioral response rank at the arm tip location among the different senescence stages (Kruskall-Wallis test, KW=7.26, p=0.044). Post-hoc Dunn's tests showed a significantly greater response intensity at the early senescent stage compared with pre-senescence (Dunn's test, p=0.028). At the base-of-arm location, although one pair-wise comparison (pre vs. mid-senescence) was significantly different (Dunn's test, p=0.045), the overall Kruskall-Wallis test for this location over time was not significant (KW=5.9, p=0.09). At the mantle test location, although trends were similar to those from the arm-tip and arm-base locations, the changes were not significant (Figure 3)

Histological analysis of arm tip tissue

There was a significant difference in total cell density (indicated by counts of DAPI-labeled nuclei in each ROI) between healthy ($n = 4$) and senescent ($n = 9$) tissues for sucker edge (unpaired t-test $p = 0.0009$; Fig. 4A-E) and axial ganglion (unpaired t-test, $p < 0.0001$; Fig. 4F-J) tissues, with tissue from senescent animals showing lower cell density. Comparisons of the epithelial tissue on the dorsal arm (unpaired t-test, $p = 0.076$; Fig 4K-O), were not significant, likely due to larger spreads of epithelial cell density for perimortem animals (see Fig. 5 for examples).

Cell density decline significantly correlated with the number of concerning, Level 4 welfare observations (Holst & Miller-Morgan, 2020) for sucker edge (simple linear regression, p = 0.0049, R^2 = 0.5630; Fig. 6A) and axial ganglion (simple linear regression, p = 0.0189, R^2 = 0.4387; Fig. 6B) tissues. Correlation of the epithelial tissue on the dorsal arm to concerning, Level 4 observations (simple linear regression, p = 0.1075, R^2 = 0.2381), were not significant, which again is likely due to the large variation in epithelial cell density for perimortem animals (Fig. 6C).

TUNEL/DAPI ratios among healthy and senescent animals remained consistent between healthy and senescent animals for all tissue types. Comparison of sucker edge (unpaired t-test, p = 0.4275, Fig 7A-E), axial ganglion (unpaired t-test, p >0.9999, Fig. 7F-J), and epithelial tissue (unpaired t-test, p = 0.9027, Fig 7K-O) were not significant.

Discussion

We show that *Enteroctopus dofleini* experiences behavioral hypersensitivity and significant deterioration of the peripheral neural and epithelial tissues during the physiological period of senescence. The behavioral changes revealed through touch tests indicate a period of hypersensitivity that begins around the time an individual is entering the reproductive phase, persists through to late senescence and then declines rapidly until the animal is almost completely unresponsive in the peri-mortem period. Likewise, we report a clear decline in the health of nervous and sucker edge tissue in the periphery through senescence, primarily indicated by progressive reduction in cell density both in neural and non-neuronal tissue.

The behavioral responses we measured were in response to two qualitatively different sensory experiences; the light von Frey filament delivered a non-noxious, possibly even sub-detection threshold stimulus to the skin, while the 26g filament was likely noxious and aversive (Bazarini and Crook, 2020; Crook et al., 2011). Although behavioral responses to the two filaments varied among animals tested in their pre-reproductive phase, in early and mid-senescence we found evidence for pronounced aversive reactions to the light filament that were similar behaviorally to responses to the heavy filament, suggesting significant reduction in activation thresholds of nociceptive neural pathways. The functional consequences of this shift toward hypersensitivity in the early reproductive phase are not clear; it is possible that these changes in sensory function serve to heighten reproductive receptivity or to enhance protective behaviors that are associated with egg care in females. The more applied implication of this finding is that even very mild dermal stimulation in animals in early senescence - when their outward appearance is quite healthy - may be perceived as aversive and thus this is a significant concern for welfare (Fig. 1).

Behavioral changes in other cephalopod species during senescence (Anderson et al., 2002; Bellanger et al., 1997; Holst and Miller-Morgan, 2020), along with declines in cognitive performance (M. P. Chichery and Chichery, 1992; R. Chichery and Chichery, 1992; Halm et al., 2000), suggest that changes to the nervous system are a result of degeneration, and may not be adaptive or functional, however, previous studies have focused on the later stages of senescence when brooding behavior is well advanced. A recent study (Wang and Ragsdale, 2018) showed changes in expression levels of neurotransmitter and other neural-function associated proteins in brooding and senescent females, indicating a prolonged and progressive suite of hormonally driven changes the nervous system occur throughout senescence. In other invertebrate species, age-associated changes to nociception and mechanosensation have been attributed to changes within sensory neurons (Ghimire and Kim, 2015), while in mammals, where the most extensive study of age-related changes to pain perception have been conducted, there is evidence for changes in both peripheral and central compartments (Devor, 1991; Lautenbacher et al., 2005; Taguchi et al., 2010).

Interestingly, we find that *E. dofleini* exhibits hypersensitivity at the very early stages of senescence. There is limited evidence for an onset of hypersensitivity during early senescence in other animals, but several studies suggest that decline in inhibitory neurotransmitter is associated with chronic pain (Yang and Chang, 2019) or neurodegenerative diseases, which often increase with age (Hou et al., 2019). Gamma-aminobutyric acid (GABA) is a conserved inhibitory neurotransmitter that modulates transmission of nociceptive signals across synapses of the central nervous system, and loss of inhibitory neurotransmitters is at least partly responsible for some aspects of chronic pain in mammals (Yang and Chang, 2019). Thus, we hypothesize the onset of hypersensitivity exhibited by *E. dofleini* at the early stages of senescence may be caused by disproportionate loss of inhibitory interneurons in the arms, resulting in hypersensitive responses to previously non-nociceptive stimulus. This could also explain observations of excessive arm-spinning during grooming, and increased movement of the arms and body in early and late senescence. Identification of neural sub-types in cephalopods is challenging, but further studies will investigate the hypothesis that loss of inhibitory control is associated with the onset of hypersensitivity in early senescence.

After early senescence, there is a clear downward trend of behavioral response from early stage to perimortem senescence. In some cases, behavioral responses completely ceased in the final few days before euthanasia. This sudden increase in response threshold after a period of hypersensitivity (abnormally low response threshold) is likely to have multiple causes, including loss of mechanoreceptor function, loss of afferent pathway integrity, or loss of motor control over withdrawal reflexes. In this study we examined tissue health only in the arms, and we also did not evaluate changes in the central nervous system, (in part due to the challenges of central brain dissections for local caretakers, compared with the relative ease of taking arm sections from euthanized animals). How the central brain declines, and how this contributes to changes in behavior, is not currently known.

It is clear that total cell density in the arms, both for neuronal and non-neuronal cell types, shows a clear downward trend as animals approach end-of-life. We had hypothesized that an increase in the rate of apoptosis and necrosis was responsible for loss of arm sensitivity at end-of-life, but unexpectedly we found no such pattern; proportions of TUNEL positive cells were the same for healthy control animals. Instead, we found greatly reduced cellular density in two of the three regions we examined (and a clear trend in the third). It is possible that the observed physiological and behavioral shift that occurs in *E. dofleini* is a result of reductions in the rate of normal cell replacement (López-Otín et al., 2013), rather than an increase in the rate of cell death.

All animals in the study were tracked by their local caretakers for outward signs of senescence, and the evaluation tool (Holst and Miller-Morgan) was used to aid euthanasia decisions. All animals in the study were euthanized; none died naturally. Thus, the physiological state at euthanasia was reflective of care-takers' decision making, and animals were euthanized in various conditions. In an effort to determine how closely external signs of decline correlated with the physiological measures of tissue health, we correlated cell density with the number of concerning, “Level 4” welfare observations reported for that animal prior to death (correlation was not possible for touch-test data since not all tissue samples came from animals with peri-mortem touch-tests). We found significant association between outward condition and cellular health in the arms, suggesting that outward measures of welfare correlate reliably with physiological tissue health.

Management of end-of-life care is of paramount importance for animal caretakers in zoos and aquariums, as well as in research labs. There has been relatively little research on welfare and euthanasia in invertebrates, but concern is growing for cephalopods in particular (Jacquet, Franks, & Godfrey-Smith, 2019; Jacquet, Franks, Godfrey-Smith, et al., 2019; Mather, 2022). The sudden drop in response thresholds in early senescence that we observed in this study suggest that proactive management and welfare assessment of senescent *E. dofleini* should begin at the onset of the reproductive phase, rather than in the terminal period. Hypersensitivity in the early senescent phase may imply that routine maintenance and handling (either in research laboratories or in public aquaria) may be perceived as aversive or painful and may have strong influence on research findings if the onset of reproductive maturity is not accounted for.

Educational facilities often tend to manage end-of-life in terminal animals by providing care that would extend the life of an animal as long as possible. However, our study indicates that cellular decline and possible loss of cellular function may lead to increased, rather than decreased, sensitivity to external stimuli that only declines as the animal enters the last days of life. Thus, efforts that focus on prolonging life until animals are extremely compromised may not be in the best interest of the animal. This study provides new evidence of a link between predictable behavioral changes during senescence in octopuses, and degeneration of peripheral tissues, and raises important new questions about sensory function, perception, and welfare of cephalopods over the course of senescence and death.

Competing Interests

Declarations

The authors have no relevant financial or non-financial interests to disclose.

Compliance with Ethical Standards

The authors have no conflicts of interest, no financial or non-financial interests that are directly or indirectly related to the work submitted for publication. This study was reviewed by the Aquarium of the Bay Research Committee to ensure ethical treatment of animals in the study.

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Table 1. Contributing institutions that either replicated behavior touch tests using von Frey Filaments (A), or provided histological arm biopsies for analysis (B). Numbers indicate the number of unique giant Pacific octopuses' behavioral datasets (A) or samples (B) provided by the respective institutions. In addition, all animals represented in the study were assessed weekly or more frequently with the GPO welfare assessment tool, which provides a measure of outward condition (Holst and Miller-Morgan, 2019). Two of the 3 samples provided by Aquarium of the Bay were biopsies of healthy, pre-senescent animals.

Contributing Institution	**Number of octopuses receiving sensory threshold testing**	**Number of octopuses providing tissue samples**
Aquarium of the Bay	4	3
Loveland Living Planet Aquarium	2	1
The Maritime Aquarium	1	1
SeaWorld San Diego	1	2
SeaWorld Orlando	0	1
Texas State Aquarium	0	1
California Academy of Sciences, Steinhart Aquarium	0	1
National Aquarium	0	1

Table 2. Stimulus response key for touch test results. After touch with one von Frey filament, the experimenter ranked the animal's response in order of ascending intensity, with whole-arm withdrawal or whole-body avoidance responses being classed as clearly nocifensive (arising from perceived noxious stimulus intensity).

Stimulus response key:
0: No response
1: <6 suction cup response
2: >6 suction cup response
3: Partial arm movement
4: Half arm movement
5: Whole arm movement
6: Multiple arm movement
7: Whole body movement

Figure Captions:

Fig 1 Giant Pacific Octopus (GPO), *Enteroctopus dofleini*. Images show the progression from pre-reproductive to terminally senescent. A. A healthy, pre-reproductive female. B &C. A female in the early stages of post-reproductive senescence. Eggs are visible in the enclosure, but the animal is still in excellent outward condition and showing largely normal behavior. D&E. Images of an animal is mid- to late senescence. The skin is beginning to lose muscle tone and color, and there are accumulating, unhealed wounds on various bodily regions. F. A terminally senescent, peri-mortem animal showing overall pale coloration, skin laxity, limpness and distal corkscrewing of the arms.

Fig 2 A micrograph of a longitudinal section through the center of the tip of one arm. For analysis of tissue degeneration we focused on three anatomically distinct arm regions; the margin of the suckers, representing a densel innervated sensory epithelium, the cortical (cell body) layer of the axial ganglia, and the dorsal skin surface, which is one of the regions where touch-tests were applied during behavioral testing. Scale bar 500uM.

Fig 3 Mechanosensory and nociceptive thresholds were measured over time in *Enteroctopus dofleini* from pre-senescence and as they declined through all stages of senescence (early, mid/late, and perimortem senescence). Results are shown for the 0.16g (non-nociceptive) and 26g (possibly nociceptive) filaments. Significant differences in mechanosensory thresholds were observed between pre- and early senescence for 0.16g at the arm tip location (p = 0.0396), but not for the base-of-arm or mantle locations. For the 26g von Frey filament, there was a significant difference at the arm tip location between pre- and early-senescence (p = 0.0275) and at the arm base location between pre- and mid/late senescence (p = 0.0451), but there were no significant changes over time at the mantle location. Bars show mean response score and error bars show SEM. Comparisons were made with Kruskall-Wallis tests for overall signifcance followed by post-hoc Man-Whitney-U tests for pairwise comparisons. Because GPO specimens entered the study at different life stages and some animals were tested inconsistently, we considered observations as independent samples for statistical analysis.

Fig 4 Comparisons of cell density in each anatomical region of the arm were compared by counting the number of DAPI puncta per three replicate ROIs, from each anatomical region, for each section. Counting was conducted using FIJI. A-E. Examples of sucker margin tissue from healthy and senescent animals shows significantly lower overall density in peri-mortem animals. White arrowheads show specific areas of tissue loss. F-J. The same comparisons shown for the axial ganglion. There was a significantly lower density of neurons in the cortical layer in senescent animals. K-O. tissue of the dorsal arm skin showed quite variable changes in terminally senescent animals (see further examples in Figure 5). Although there was evidence of reductions in this region too, the comparison was not significant.

Fig 5 Examples of tissue samples from the dorsal epithelial region in two different peri-mortem animals (A&B) and a healthy control (C). In A, there is almost no evidence of remaining epithelial tissue, and instead the underlying muscle is exposed, which is also in poor condition. Loss of skin at the arm tips is common in peri-mortem senescence. B. This animal has an unusual, high-density aggregation of atypical cells under the skin surface. We did not attempt to characterize these cells. They may be an aggregation of hemocytes. C. Dorsal epithelium from a healthy control sample shows clear tissue boundaries and high density of cells.

Fig 6 Octopuses in this study were all euthanized by their care team at their home institutions, and euthanasia decision making is variable. Here, we evaluated whether there was a relationship between tissue health at euthanasia and the number of “high concern” welfare indicators recorded for that animal prior to death, as a proxy for external condition. There was a significant association between external measures of decline and cellular degeneration in the sucker edge (A) and ganglion (B), but not the dorsal arm skin.

Fig 7 Comparisons of the ratios of TUNEL-positive to DAPI-positive cells in each anatomical region of the arm we counted by dividing the number of cyan puncta by the number of blue puncta. Green labeling that was not co-localized with blue was excluded (such as the strongly auto-fluorescent cells of the sucker cup inner edge, visible in C). Counts were conducted using FIJI. A-E. Examples of sucker margin tissue from healthy and senescent animals showed relatively high levels of TUNEL-positive cells in both the healthy control and the terminally senescent animal. F-J. The same comparison shown for the axial ganglion, again showing no significant difference in TUNEL staining. K-O. Comparisons of the dorsal skin surface also showed no indication of increased rates of cell death at terminal senescence.