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A Morphological Study of Nonrandom Senescence in a Colonial Urochordate

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Abstract. *Botryllus schlosseri* is a clonally modular ascidian, in which individuals (zooids) have a finite life span that is intimately associated with a weekly budding process called blastogenesis. Every blastogenic cycle concludes with a synchronized phase of regression called takeover, during which all zooids in a colony die, primarily by apoptosis, and are replaced by a new generation of asexually derived zooids. We have previously documented that, in addition to this cyclical death phase, entire colonies undergo senescence during which all asexually derived individuals in a colony, buds and zooids, die in concert. In addition, when a specific parent colony (genet) is experimentally separated into a number of clonal replicates (ramets), ramets frequently undergo senescence simultaneously, indicating that mortality can manifest itself in nonrandom fashion. Here, we document a morphological portrait of senescence in laboratory-maintained colonies from Monterey Bay, California, that exhibit nonrandom mortality. Nonrandom senescence proceeded according to a series of characteristic changes within the colony over a period of about one week. These changes included systemic constriction and congestion of the vasculature accompanied by massive accumulation of pigment cells in the zooid body wall (mantle), blood vessels, and ampullae; gradual shrinkage of individual zooids; loss of colonial architecture, and ultimately death. At the ultrastructural level, individual cells exhibited changes typical of ischemic cell death, culminating in necrotic cell lysis rather than apoptosis. Collectively, these observations indicate that senescence is accompanied by unique morphological changes that occur systemically, and which are distinct from those occurring during takeover. We discuss our findings in relation to current experimental models of aging and the possible role of a humoral factor in bringing about the onset of senescence.

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Introduction

The colonial urochordate *Botryllus schlosseri* (Tunicata, Ascidiacea) is a ubiquitous, filter-feeding inhabitant of shallow waters and harbors worldwide, where it is found on a wide variety of hard substrates (Sabbadin, 1969). Each colony arises from a sexually produced, chordate larva that attaches itself to a substratum and undergoes metamorphosis. The resulting oozooid immediately begins to produce buds that originate as outgrowths from its lateral wall and are genetically identical to the parent. Within about one week between 16° and 20°C, the oozooid regresses and the first asexual generation of buds simultaneously reaches functional maturity (Sabbadin, 1969). This first generation of blastozooids produces its own buds, which in turn mature in about a week and subsequently die after giving rise to an additional set of zooids. Each such cycle, called blastogenesis, is entirely temperature-dependent and of shorter duration at higher temperatures (Milkman, 1967; Sabbadin, 1969; Rinkevich *et al.,* 1998). During the last stage of a typical blastogenic cycle (the takeover stage), all zooids in a colony regress over a 24- to 30-h period through the death of all visceral tissues, primarily by apoptosis (Lauzon *et al.,* 1992, 1993). Thus, the life history of a *B.*

schlosseri colony is characterized by a long series of such blastogenic cycles, which succeed one another periodically. In time, a colony can grow up to hundreds of genetically identical blastozooids, with all zooids and buds in the colony being developmentally synchronized (Milkman, 1967). Zooids are typically arranged in star-shaped groups called systems, which are embedded in a gelatinous tunic. Blood continuously flows throughout the systems, zooids, and buds, and into the peripheral endings of blood vessels called ampullae.

In addition to this weekly death process, individual colonies have finite life spans. Colonies grown under field conditions are characterized by short, sub-annual life spans (Berrill, 1950; Millar, 1952; Brunetti, 1974; Brunetti and Copello, 1978; Grosberg, 1988; Chadwick-Furman and Weissman, 1995a, b). For instance, in *B. schlosseri* colonies from Monterey Bay, maximum life span ranges from about 3 months for animals born in the spring to just over 8 months for those born in the fall (Chadwick-Furman and Weissman, 1995a). Senescence appears to be the primary cause of mortality in field-raised colonies, and involves a series of characteristic degenerative changes that culminate in the death of the entire colony (Brunetti, 1974; Chadwick-Furman and Weissman, 1995a). In contrast, laboratory-maintained colonies from Monterey Bay have been documented to exhibit life spans of more than 7 years (Rinkevich and Shapira, 1998), and counterparts from the Venetian Lagoon (Italy) were reported to have been kept alive for up to 3 years (Sabbadin, 1969). It is well documented that this species exhibits tremendous plasticity, and that life-history traits are subject to variation by changes in environmental conditions (Chadwick-Furman and Weissman, 1995a, b; Rinkevich *et al.,* 1998). For instance, colonies grown under field conditions attain large sizes, form compact structures, and reproduce rapidly, whereas laboratory-maintained colonies grow slowly, often fragment into subcolonies, and may not undergo sexual reproduction (Chadwick-Furman and Weissman, 1995b). Yet, it is precisely in the laboratory environment that this animal can best be manipulated and studied as a model system of invertebrate immunity (reviewed in Magor *et al.,* 1999), development (Milkman, 1967; Lauzon *et al.,* 1992; Rinkevich *et al.,* 1992; Lauzon *et al.,* 1993), cell and molecular biology (DeTomaso *et al.,* 1998), and evolutionary studies (Stoner *et al.,* 1999). We previously reported that laboratory colonies (genets) and the clonal replicates (ramets) derived from these genets underwent regressive changes at the end of their life span that were unlinked to reproductive effort (Rinkevich *et al.,* 1992). These changes often led to the death of previously separated ramets from a given genet simultaneously within the same blastogenic cycle. The nonrandom nature of this process strongly suggested that senescence could be mediated by a heritable component (Rinkevich *et al.,* 1992). The findings reported in this paper extend our previous observations indicating that senescence is reproducibly associated with characteristic morphological changes that are conserved from colony to colony and appear identical to those reported in field colonies. Furthermore, since field studies have only described macroscopic changes occurring at the colony level (Brunetti, 1974; Chadwick-Furman and Weissman, 1995a), it has become imperative to document cellular and subcellular features occurring during nonrandom senescence, as a means of understanding its underlying mechanism. Here, we provide evidence that the histological and ultrastructural features of cell death during nonrandom senescence are distinct from those occurring during takeover and ultimately reflect systemic changes within the colonial vasculature.

Materials and Methods

Laboratory mariculture of Botryllus schlosseri

Animals were maintained between 16° and 17°C under laboratory conditions, as previously described (Boyd *et al.,* 1986; Rinkevich *et al.,* 1992). In our initial study on senescence and mortality in this species, we had observed that senescence in laboratory-maintained colonies could occur in either a random or a nonrandom fashion (Rinkevich *et al.,* 1992). To document the morphological changes that are consistently associated with senescence, we have focused solely on the latter type. For the animals reported in this study, individual colonies (genets) were separated with the use of a razor blade into several clonal replicates (ramets). Each ramet was attached to a slide, after which they were maintained separately or within the confines of the same tank. Our previous findings had indicated that, within the group of colonies exhibiting nonrandom senescence, the placement of ramets within the same or different tank did not affect the onset of senescence. That is, ramets derived from the same genet underwent simultaneous degenerative changes that led to their death, regardless of whether they were placed in the same tank or kept in separate tanks (Rinkevich *et al.,* 1992). Dates of subcloning, colony number, and numbers of ramets were recorded as previously described (Rinkevich *et al.,* 1992). Life span was recorded as number of blastogenic cycles until death. Observations of colonies and determination of zooid length were carried out with the use of a stereomicroscope (Stemi SV 6, Carl Zeiss, Germany) equipped with an ocular micrometer.

Transmission electron microscopy

Colonies undergoing nonrandom senescence were separated into individual systems (star-shaped groups of zooids) with a razor blade on the third day after the onset of systemic vasculature constriction and increased pigmentation of zooids. Day 3 was selected as representative of an

early stage of senescence. The systems were processed for electron microscopy as previously described (Lauzon *et al.*, 1993). In brief, the systems were fixed in a solution of 1% glutaraldehyde, 1% acrolein (freshly distilled), and 80% artificial seawater (ASW) for 2 h at room temperature. Individual systems were washed several times in ASW for a total of 1 h and were then post-fixed in 1% osmium tetroxide for 1 h in ASW at room temperature. The specimens were subsequently dehydrated in a graded acetone series and embedded in Spurr's resin (Polysciences, Warrington, PA) according to the manufacturer's specifications. Ultrathin sections were cut with a diamond knife (EM Corp, Chestnut Hill, MA), placed onto Formvar-coated 300-mesh nickel grids, stained with 2% aqueous uranyl acetate and 1% lead citrate, and examined with a Phillips EM 201 transmission electron microscope.

Histology

Individual systems from senescent colonies (days 3 and 5) were isolated with the use of a razor blade and fixed in 1% paraformaldehyde-filtered seawater for 2 h at 4°C. Days 3 and 5 were selected as representative of early and middle senescence respectively. Specimens were subsequently dehydrated in a graded ethanol series, and embedded in JB-4 plastic (Polysciences, Warrington, PA) according to the manufacturer's specifications. Sections ranging between 2 and 3 μm in thickness were generated with a Histoknife (Diatome Inc., Switzerland) along the anteroposterior axis of zooids, as previously described (Lauzon *et al.*, 1992). Individual sections were stained with 0.01% toluidine blue-sodium bicarbonate, mounted with Permount (Fisher Scientific, Springfield, NJ), and observed under bright-field microscopy (Olympus BH-2 model, New York/New Jersey Scientific, Inc., Middlebush, NJ).

Results

Senescence is a heritable process in some Botryllus schlosseri *colonies*

In our previous study of senescence in *B. schlosseri* colonies maintained under laboratory mariculture, 17 of 41 genets were documented to exhibit nonrandom mortality (Rinkevich *et al.*, 1992). The life spans of some additional laboratory-born and raised colonies were determined by following animals until the entire colony manifested senescent features indicative of impending death. Eleven genets were selected on the basis of their ability to undergo nonrandom mortality. As illustrated in Figure 1, death was synchronous between all ramets in 7 of 11 colonies (Y8, 275jjr, 403d, 386i, 433z, 435h, 360c), occurring within the same blastogenic cycle. In 3 of the remaining 4 colonies (Y7, 248hsi and 433af), all ramets died simultaneously, with the exception of one ramet from each of the genets,

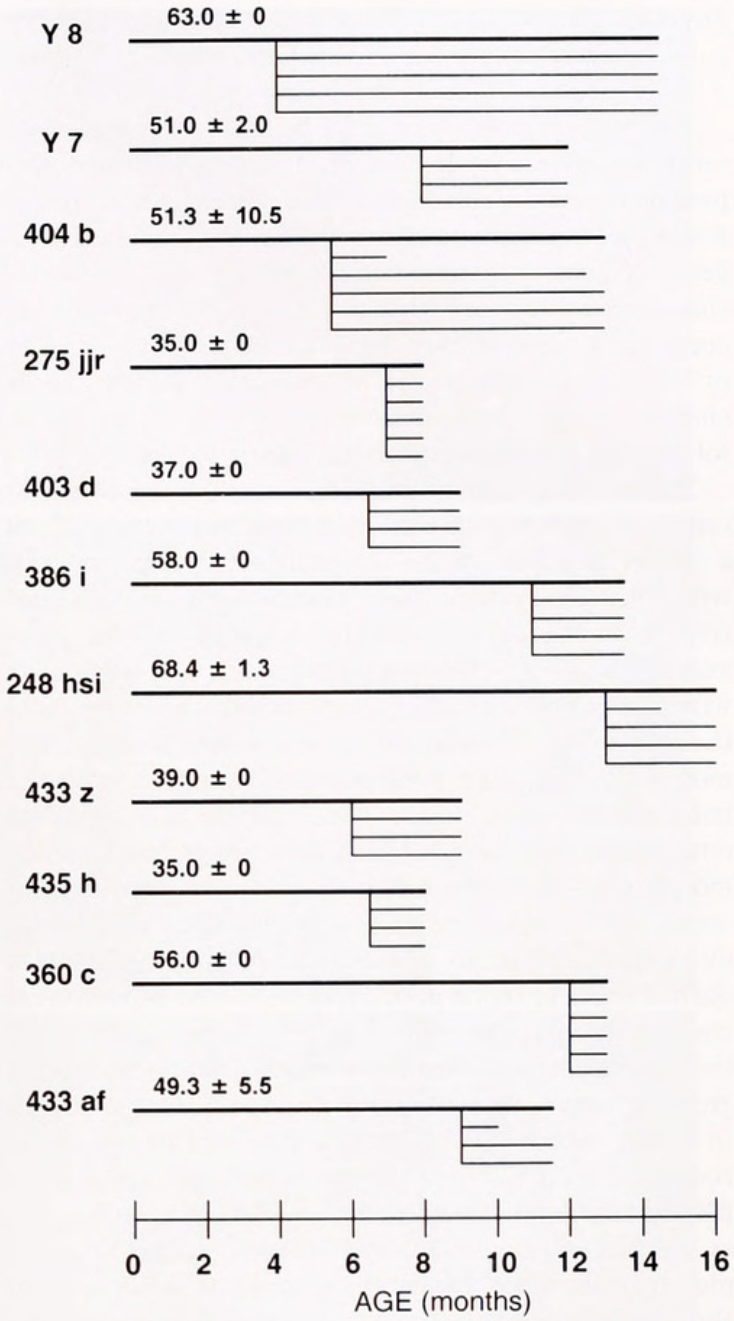

Figure 1. Lifespan of "parent" *Botryllus schlosseri* colonies and derived clonal replicates that express nonrandom mortality. The genet numbers are indicated on the left margin. The lifespan of the original parent part is indicated by a thick horizontal line, whereas lifespan from subcloned ramets derived from the original parent part is indicated by a thin line. When multiple ramets were subcloned on a given day, their lifespans are indicated by parallel horizontal lines originating from a common perpendicular line. The numbers above each thick line represents the mean number of blastogenic cycles until death ± standard deviation (SD) for each genet. Each blastogenic cycle lasts 7 days at 16°–17°C under laboratory conditions.

which died either 4 (Y7) or 6 (248hsi and 433af) blastogenic cycles earlier. In colony 404b, two ramets died before the others—one 2 blastogenic cycles earlier and the other 24 cycles earlier (in this ramet, death was probably unrelated to senescence).

Stereomicroscopic observations of Botryllus schlosseri *colonies undergoing nonrandom senescence*

The findings in this report are based upon repeated observations, over several years, of characteristic changes that preceded death of individual colonies. Because the onset of senescence could not be accurately predicted within a given genet or ramet, colonies were observed daily for these characteristic changes. When observed under stereomicroscopy, ramets derived from the 11 genets displayed a series of regressive changes over a one-week period. These changes resulted in the near-simultaneous death of a genet following a given number of blastogenic cycles.

The earliest recognizable features of nonrandom senescence involved the constriction of blood vessels throughout a colony, resulting in the accumulation of pigment cells within the zooid mantle, buds, blood vessels, and ampullae (Fig. 2). In nonsenescent colonies, ampullae serve as reservoirs for pigment cells during takeover, and thus accumulate within them only during this phase of the blastogenic cycle (Lauzon *et al.,* 1992). In senescent colonies, however, this morphological feature was consistently observed to occur independently of takeover. The ampullae also began to retract from the leading edge of the colony, perhaps as an indication of declining health (Fig. 2b). At this stage of senescence, zooids were still actively feeding, as assessed by the functional nature of both oral and excurrent siphons, but had begun to shrink in size relative to their nonsenescent counterparts. The average zooid length for randomly selected zooids from nonsenescent colonies during the asexual growth phase of the blastogenic cycle was 2.34 mm $\pm$ 0.29 ($n = 85$), compared to 1.69 mm $\pm$ 0.15 for early senescent zooids ($n = 105$; $P < 0.05$ by paired Student's t test). Blood flow slowed considerably between days 1 and 3, and as a result of heavy congestion of the vessel walls with pigment cells, blood circulation became progressively more sluggish between days 3 and 6 of nonrandom senescence. The first vessels to be affected were the marginal vessels near the periphery of the colony. Buds were also affected during this process: they gradually separated from the parent zooids (data not shown), eventually becoming developmentally arrested. By day 6 of nonrandom senescence the systems had become highly disorganized, with concomitant loss of the common excurrent siphon, and zooids had separated from one another (Fig. 2c). Zooids at this degenerative stage were further shrunken (1.01 mm $\pm$ 0.17, $n = 102$; $P < 0.01$, by paired Student's t test), displayed a more intense pigmentation pattern, and were scattered individually in the tunic or formed small groups of two to three zooids. In addition, the hearts of individual zooids exhibited weak and infrequent pulsations, signaling impending death. Between days 3 and 6 of nonrandom senescence the oral and excurrent siphons became progressively more dilated (Fig. 2b, c). Zooids gradually lost sensitivity to mechanical stimulus. By day 7, all zooids in the colony were dead, as assessed by lack of heartbeat and shutdown of the siphons (Fig. 2d). At day 9, the colony was in an advanced state of decay and zooids were no longer recognizable. Protozoa were observed swimming on the surface of the softened tunic, presumably contributing to the decay process (data not shown).

In some instances, the onset of senescence was also observed during the takeover phase of the blastogenic cycle. Takeover is characterized by the simultaneous regression of the previous generation of zooids and the increase in size and maturation of the new generation of pre-functional zooids. In nonsenescent colonies, takeover begins with the shutdown of the oral and excurrent siphons, followed by polarized zooid contraction; it is generally completed within 24 to 30 h between 16°–18°C (Lauzon *et al.,* 1992). At the conclusion of takeover, the zooid remnants appear as small vesicles, and a new blastogenic cycle begins with the opening of oral and excurrent siphons in the next generation of zooids (Lauzon *et al.,* 1992). In contrast to the process seen in nonsenescent colonies, zooids were never completely resorbed in colonies undergoing nonrandom senescence. In a representative colony at day 5 of the senescent process, the previous zooid generation was still visible 60 h after the onset of takeover, although considerable resorption had already taken place (Fig. 2e). At this stage, the next asexual generation of zooids had open oral and excurrent siphons but exhibited signs of fragmentation, in that they failed to form complete star-shaped systems. Instead, zooids were found individually or assembled into groups of two or three with a common excurrent siphon. In addition, the systemic vasculature had become sluggish, and pigment cells—which normally leave the ampullae at the conclusion of takeover—remained within them. At 110 h after takeover, the entire colony was dead, with resorption products still visible (Fig. 2f).

Histological observations of nonrandom senescence

Histological studies have previously been carried out on nonsenescent zooids during blastogenic growth (Lauzon *et al.,* 1992). In contrast to those nonsenescent zooids, individual zooids at day 3 of nonrandom senescence prominently displayed congested visceral tissues and vascular spaces (Fig. 3a). A large proportion of cells from visceral tissues also appeared swollen with a hollow appearance. These cells invariably took up very little stain (weakly basophilic) and were at times harboring pyknotic nuclei. In some cases, cells with large, open spaces suggestive of cell lysis were also found. At this stage of senescence, visceral tissues (Fig. 3a, b, for stomach, intestine, endostyle; pharyngeal slits and neural complex not shown) were still confined by an intact perivisceral epithelium, and thus few cells were free in the peribranchial cavity (Fig. 3b). In

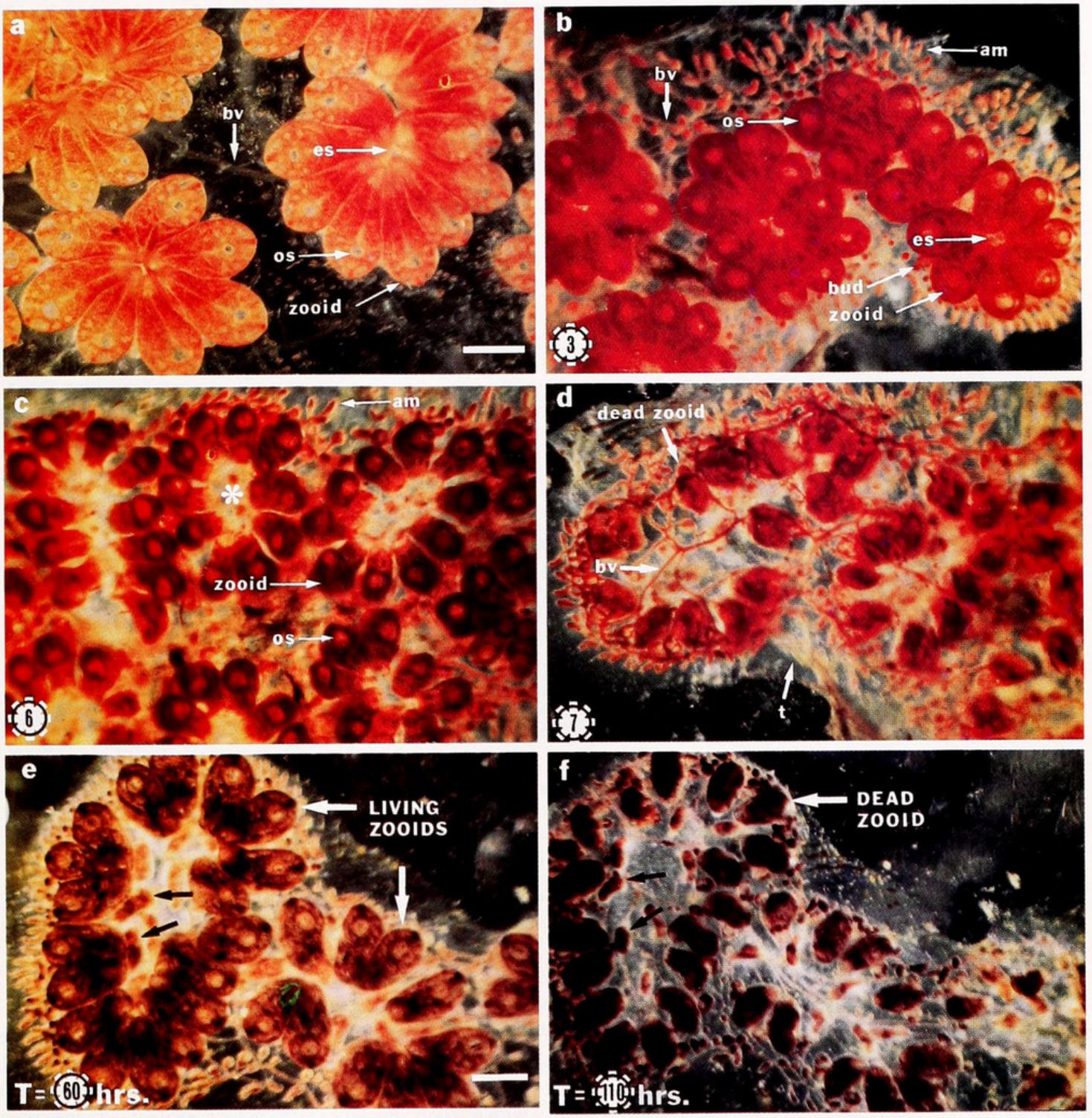

Figure 2. *Botryllus schlosseri* colonies at different stages of nonrandom senescence. (a) Appearance of star-shaped groups of zooids in a nonsenescent colony. (b) A representative colony 3 days following the onset of senescence. Note the dark pigmentation pattern within zooids and accumulation of pigment cells in blood vessels and ampullae. (c) At day 6 of nonrandom senescence, there is loss of colonial architecture. The zooids within individual systems have disconnected from one another following loss of the common excurrent siphon. Note that the siphons from individual zooids have greatly dilated. Systemic blood flow has almost shut down except for the central part of the colony. (d) The end stage of senescence (day 7) is characterized by complete stasis of the colonial vasculature and dead zooids with closed siphons. (e) A different colony at day 5 of nonrandom senescence, observed at 60 hours following the onset of takeover (bottom left corner). Note that the zooids of the previous asexual generation are still visible (black arrows).(f) The same colony observed at 110 hours following onset of takeover, depicting dead zooids with resorption products still visible (black arrows). Panels a–c and d represent dorsal and ventral views respectively of zooids from different portions of the same colony (orange color morph) undergoing nonrandom senescence. Panels e–f are dorsal views of the same part of a different colony (blue/brown color morph). The numbers at the bottom left portion of panels a–d represent the days following onset of nonrandom senescence. Abbreviations: am, ampulla; bv, blood vessels; es, excurrent siphon; os, oral siphon; t, tunic. Asterisks represent the region occupied by the common excurrent siphon. Scale bars equal 1 mm for panels a–d and e–f respectively.

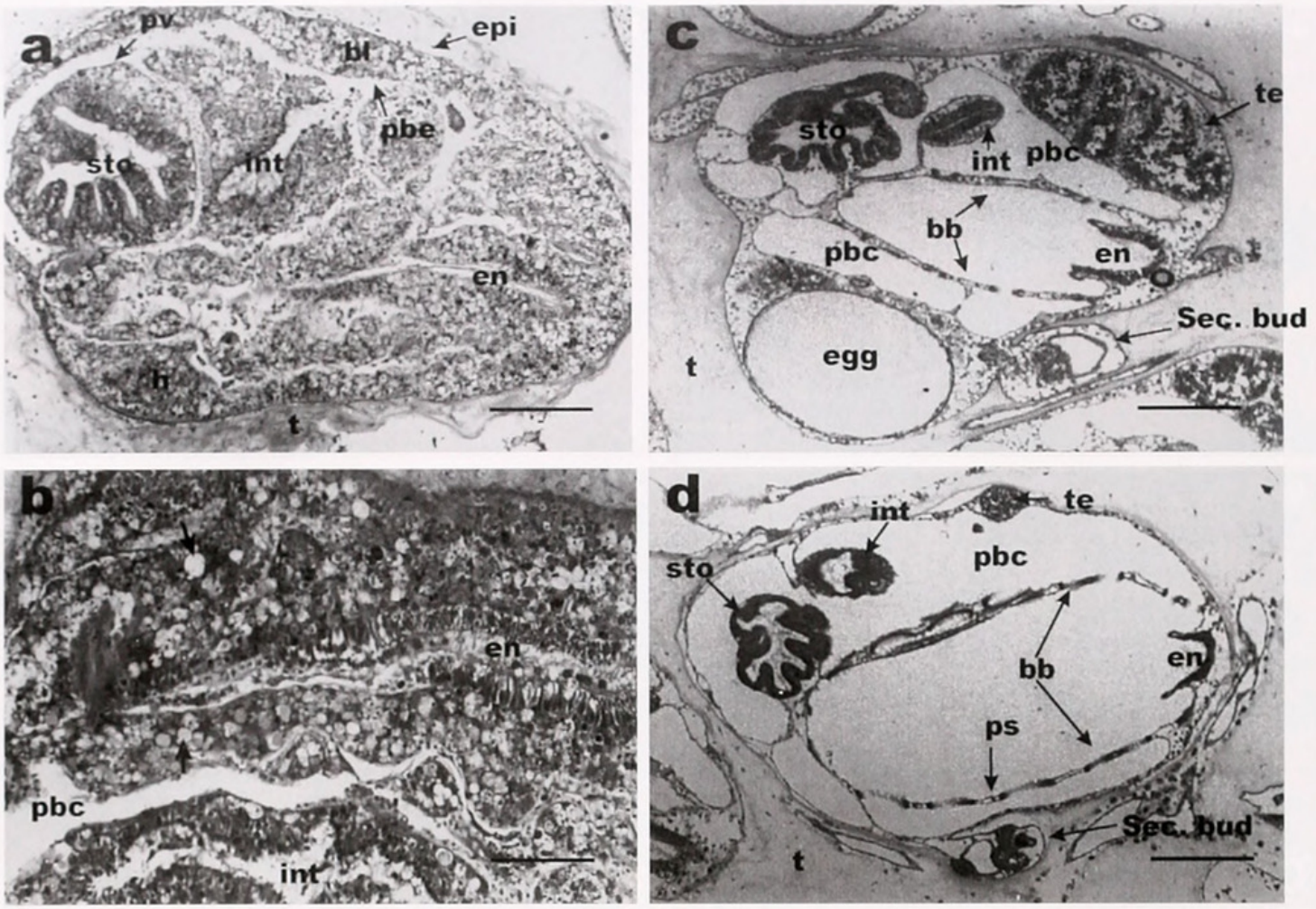

Figure 3. Histological observations of *Botryllus schlosseri* zooids and buds at day 3 of nonrandom senescence. (a) Ventral section of a senescent zooid. At this stage, zooids are congested and visceral organs have a spongy appearance, characterized by large empty spaces or swollen cells with weakly basophilic cytoplasm. (b) Higher magnification of the endostyle near the ventral sinus depicting the swollen cells that are weakly basophilic (large black arrows). Cells with multiple nuclei are widespread along the anteroposterior axis of the zooid, indicative of extensive phagocytosis of dying cells. (c) Primary and secondary buds of a day 3 senescent colony appear morphologically normal as compared with buds from a nonsenescent colony (d). Abbreviations: bb, branchial basket; bl, blood; en, endostyle; epi, epidermis; h, heart; int, intestine; pbc, peribranchial cavity; pbe, peribranchial epithelium; ps, pharyngeal slits; pv, perivisceral epithelium; sec. bud, secondary bud; sto, stomach; t, tunic; te, testes. Scale bars = 240 μm (a, c, d) and 120 μm (b).

contrast to the features associated with zooids at day 3 of senescence, primary and secondary buds were morphologically indistinguishable from buds of nonsenescent animals (Fig. 3c, d).

By day 5 of senescence, the squamous perivisceral and peribranchial epithelia and the epidermis of zooids was visibly swollen, thus exhibiting degenerative changes similar to those observed for other cells at day 3 (Fig. 4a). These changes presumably led to the release of individual cells or groups of cells from the viscera or blood into the peribranchial cavity (Fig. 4a). In contrast, filter-feeding zooids from nonsenescent animals retained intact squamous perivisceral and peribranchial epithelia (Fig. 4b). Throughout the progression of senescence, as blood vessels constricted and blood flow became sluggish, pigment cells and other blood cells accumulated within ampullae, thus conferring a congested appearance (Fig. 4c) not seen in nonsenescent colonies during blastogenic growth (Fig. 4d). In addition, phagocytic cells containing many ingested cells with pyknotic nuclei became more prevalent over the course of senescence (Fig. 4a, c). By day 5, the buds began to exhibit morphological degeneration similar to that seen in zooids two days earlier, and the colonies usually died at day 7, often loaded with trapped eggs and sperm (data not shown).

Ultrastructural observations of nonrandom senescence

The ultrastructure of senescent zooids revealed a number of features diagnostic of ischaemic cell death with cellular necrosis as an end-point; these supported the histological profile seen by light microscopy. At day 3, small groups of

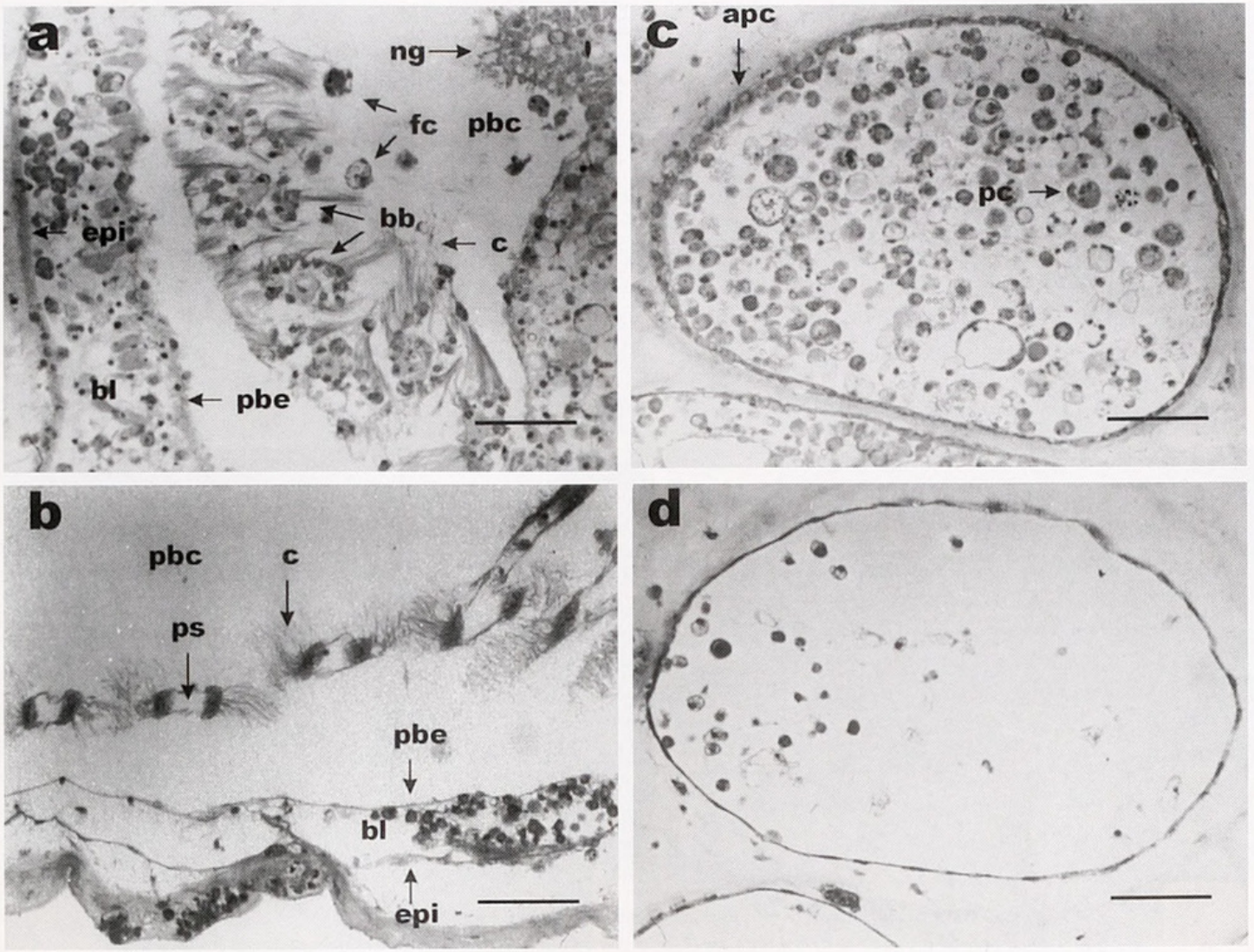

Figure 4. Histological observations of zooids at day 5 of nonrandom senescence. (a) Dorsal section of a day 5 senescent zooid near the neural complex, outlining the progressive disorganization of the pharyngeal slits within the branchial basket, and the shedding of individual cells or groups of cells into the peribranchial cavity. (b) Pharyngeal slits in a comparable dorsal section from a nonsenescent colony. Epithelia (peribranchial epithelium and epidermis) from nonsenescent zooids are squamous, whereas during senescence, the epithelium swells. (c) Cross-section through a peripheral ampulla from a senescent colony at day 5 of senescence. Note the congested appearance of blood cells within the ampulla as compared to an ampulla from a nonsenescent colony (d). Abbreviations: apc, ampullar pad cell; bb, branchial basket; bl, blood; c, cilia; epi, epidermis; fc, free cells in peribranchial cavity; ng, neural gland; pbc, peribranchial cavity; pbe, peribranchial epithelium; pc, phagocytic cell; ps, pharyngeal slits. Scale = 60 μm.

cells interspersed within visceral tissues began a sharp decrease in cytoplasmic density accompanied by pronounced distension of the endoplasmic reticulum (Fig. 5a, b: branchial basket; Fig. 5c, d: intestine). Within these cells, the inner and outer leaflets of the nuclear envelope separated, but the chromatin configuration remained nearly normal as compared with unaffected neighboring cells (Fig. 5a, d). The nuclear hallmarks of apoptosis, such as chromatin condensation and margination against the nuclear envelope, were not observed in the senescent tissues examined by electron microscopy (Fig. 5: branchial basket and intestine; Fig. 6: endostyle and stomach). Another change associated with the nonrandom senescent phenotype was a prominent Golgi apparatus, seen primarily in those cells in which cytoplasmic density was markedly reduced. In contrast, the plasma membrane remained intact at this time, and contacts with neighboring cells were maintained (Fig. 5a–d).

During the takeover phase of blastogenesis, visceral tissues from the zooid undergo apoptotic cell death, and mitochondrial architecture is generally preserved throughout this process (Lauzon *et al.*, 1993). However, examination of the mitochondria during nonrandom senescence revealed that they too underwent pronounced degenerative changes, appearing swollen, with their cristae both distended and disorganized (Fig. 5b–d). More importantly, these changes frequently preceded the loss of cytoplasmic density (Fig. 5a,

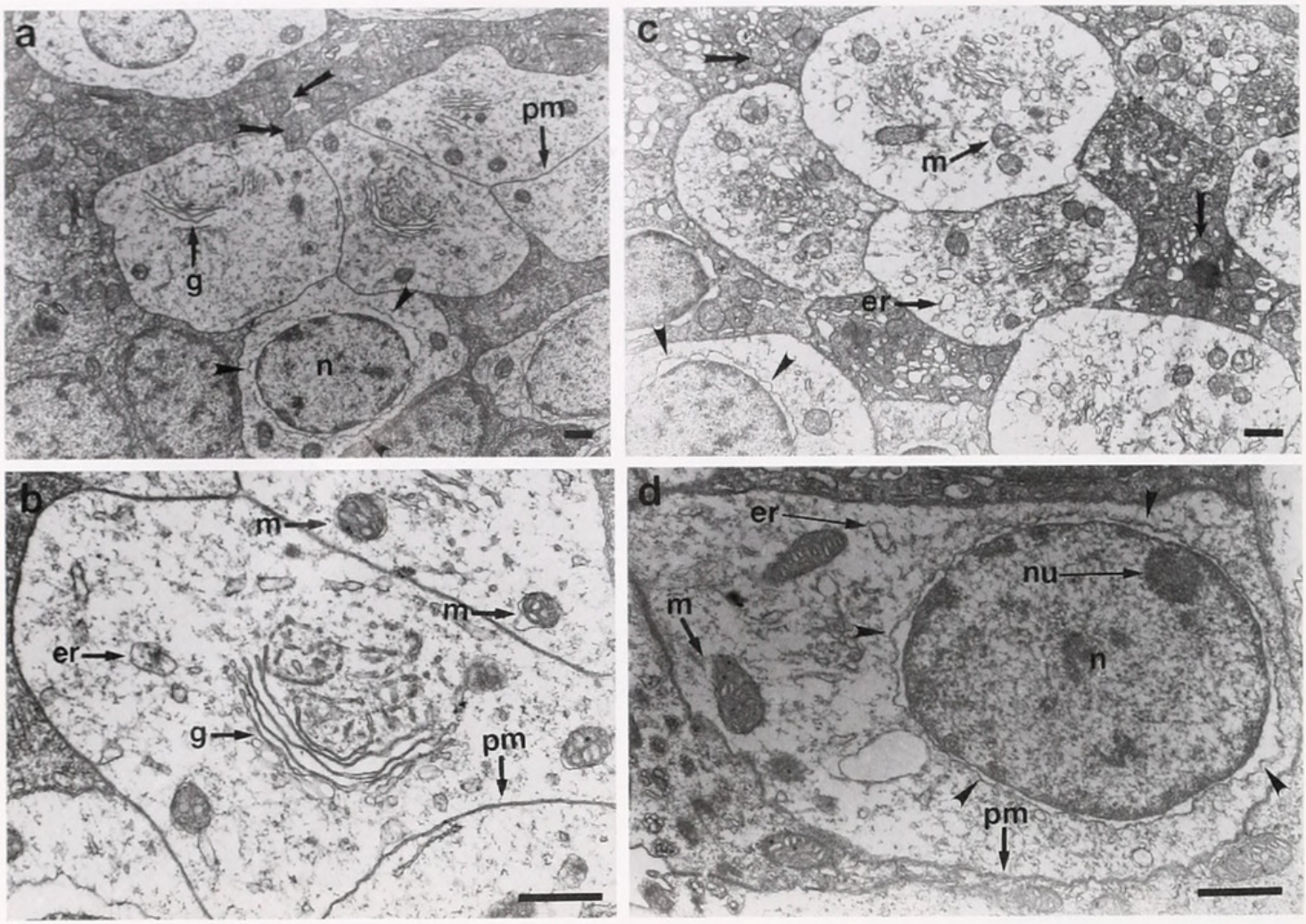

Figure 5. Ultrastructural analysis of visceral cell death at day 3 of nonrandom senescence. Cross-sections through the branchial basket (a, b) and intestine (c, d) at day 3 of nonrandom senescence. Ultrastructural features are diagnostic of ischemic cell death, and include the separation of the nuclear envelope (black arrowheads in a, c, and d), a pronounced decrease in cytoplasmic density (a–d), distension of the endoplasmic reticulum (b–d), swelling of mitochondria with concomitant distension and disorganization of cristae (b–d), and appearance of a prominent Golgi apparatus (a, b). Note that mitochondrial disruption is also observed in cells with normal cytoplasmic density (large black arrows in a, c). In contrast, the chromatin appears normal at this stage, and the plasma membrane remains intact (a–d). Abbreviations: er, endoplasmic reticulum; g, Golgi apparatus; m, mitochondria; n, nucleus; nu, nucleolus; pm, plasma membrane. Scale bars = 1 μm.

c). Interestingly, this degeneration did not occur simultaneously in all cells of a given visceral tissue (Fig. 5a) or other tissues (Fig. 6b, d). Other ultrastructural changes that were observed at day 3 of nonrandom senescence included accumulation of large intra-cytoplasmic lipid inclusions throughout cells of the viscera and blood (Fig. 6a), fusion of microvilli at the brush border of the digestive epithelium (Fig. 6b), and extensive phagocytosis of dying cells throughout the anteroposterior axis of the zooid (Fig. 6c). In addition, lysosomal activity was inferred to be an important component in cell death in some instances, as autophagosomes were frequently observed within cells of the endostyle near the ventral sinus, independently of the necrotic changes described above (Fig. 6d).

Discussion

We have previously documented the longevity of 41 independent, laboratory genets and observed that senescence in *Botryllus schlosseri* could manifest itself in either random or nonrandom fashion (Rinkevich *et al.*, 1992). During nonrandom senescence, specific parental colonies and their subcloned ramets died within a defined period of time, often within the same blastogenic cycle. Here, we have described a morphological profile of nonrandom senescence at the level of the genetic individual, the genet. Under laboratory conditions, specific colonies had finite life spans, and most of the ramets from a given genet exhibited in-concert, degenerative changes that signaled imminent death within about 7 days. The nonrandom senescent phe-

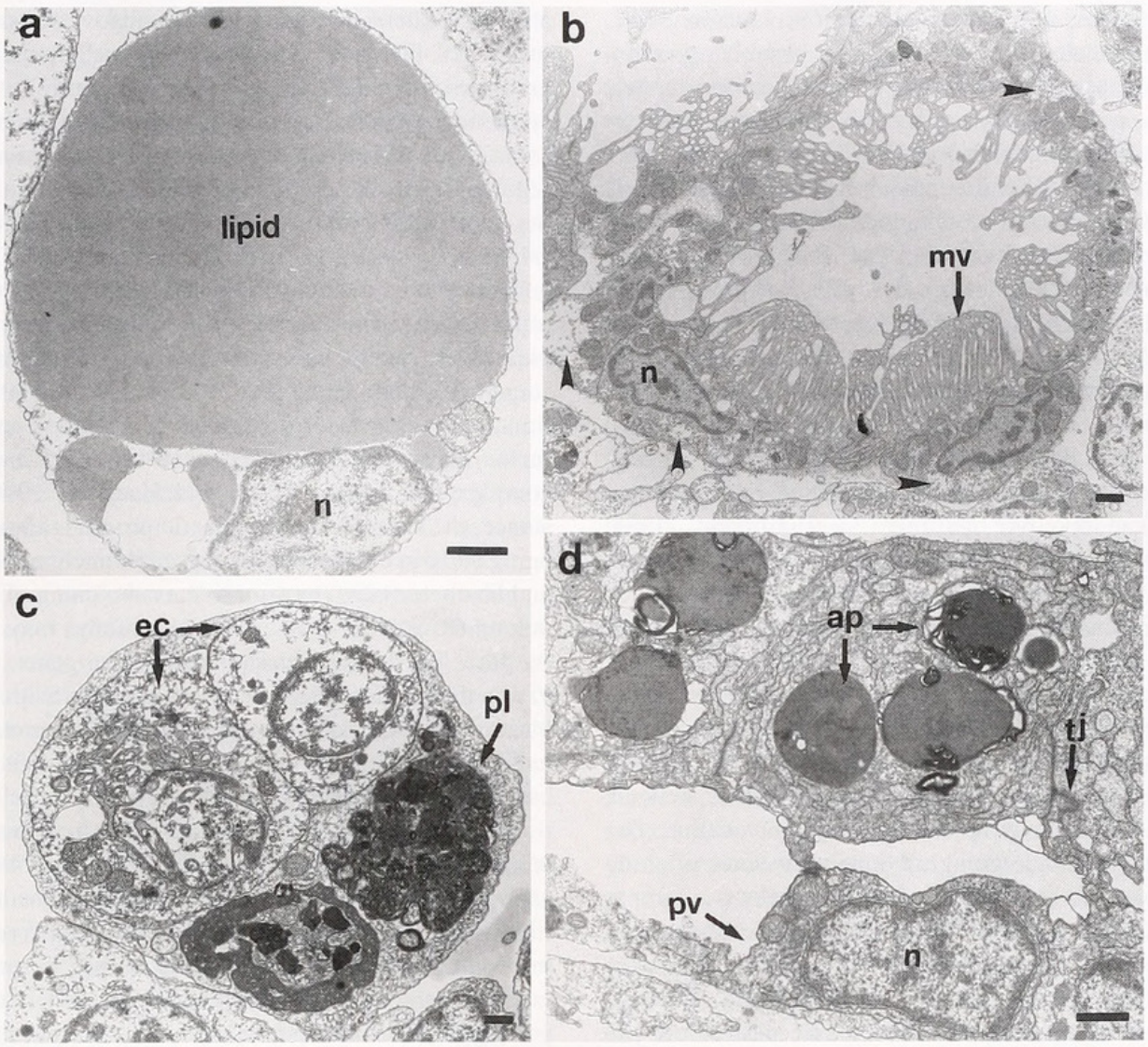

Figure 6. Further ultrastructural alterations in zooids at day 3 of nonrandom senescence. (a) Individual cell with prominent lipid inclusion found in blood space of zooid. Such cells are found randomly dispersed in blood and viscera throughout the dorsoventral plane of individual zooids. (b) Cross-section through the brush border of the gastric epithelium reveals fused microvilli. These cells do not yet display any disruption of the nuclear membrane, but some loss of cytoplasmic density can be observed (large black arrowheads). (c) Phagocyte found in the branchial basket with multiple phagolysosomes. (d) Longitudinal section of the endostyle, near the ventral sinus, reveals prominent membrane whorls indicative of autophagy. However, the tight junctions have remained intact. Abbreviations: ap, autophagic vacuoles; mv, microvilli; ec, engulfed cell; n, nucleus; pl, phagolysosome; pv, perivisceral epithelium; tj, tight junctions. Scale bars = 1 μm.

notype in this species was defined by characteristic morphological changes that were identical from colony to colony, and which began with the systemic constriction of the colonial vasculature, followed by cellular necrosis as an end-point. Macroscopic, senescence-associated changes similar to the ones reported here have been documented in field-grown *B. schlosseri* colonies (Brunetti, 1974; Chadwick-Furman, 1995a). For instance, Brunetti (1974) described a mortality process with rapid onset, in *B. schlosseri* colonies from the Venetian Lagoon (Italy). The features of this process included slowing of blood flow; storage of pigment cells within blood vessels, ampullae, and zooid body wall; inability of zooids to form complete systems; dilation of siphons; and separation of buds from the parent zooids. The concordance between our macroscopic observations of laboratory colonies and those resulting from field work are important because this species exhibits tremendous plasticity of life-history traits in response to environmental variation (Brunetti, 1974; Chadwick-Furman and Weissman, 1995b; Rinkevich *et al.,* 1998). Furthermore, our studies are the first to document in detail the morphology of senescence at the cellular and subcellular levels in this species.

In the clonally modular ascidian *B. schlosseri,* death processes can be investigated at three levels: the individual zooids, the ramets, and the genet. Studies on individual

zooids (Milkman, 1967; Sabbadin, 1969; Lauzon *et al.,* 1992) have revealed the existence of a weekly, synchronized cycle of asexual development and regression, known as the blastogenic cycle, which concludes with massive visceral apoptosis (Lauzon *et al.,* 1993). On the other hand, senescence at the level of the ramet has not been extensively investigated. In contrast to nonrandom senescence, senescence at the ramet level can manifest itself at random, and thus may occur at any blastogenic cycle, independently of the genet (Rinkevich *et al.,* 1992). This process also exhibits a characteristic morphology; this includes synthesis of fewer than one bud or zooid per blastogenic cycle, constriction of blood vessels, accumulation of pigment cells within zooids and ampullae, dispersal of zooids within systems, degeneration of the tunic and loss of zooid resorption potential during takeover (Brunetti and Copello, 1978; Rinkevich *et al.,* 1998). These features resemble nonrandom senescence documented in *B. schlosseri* genets (Rinkevich *et al.,* 1992; this study). There are, however, two major differences between nonrandom and random senescence. First, nonrandom senescence is fast, being completed in 7 days from onset of morphological changes, as compared to the weeks and months of gradual deterioration that is typically associated with random senescence of ramets. Second, random senescence may be followed by rejuvenation. Degenerating ramets undergoing random senescence often do not die (Rinkevich *et al.,* 1998). Several weeks (sometimes more than 2 months) after the manifestation of senescent features, colonies may return to a fast growth rate characteristic of young colonies (Rinkevich *et al.,* 1998). Although senescence is regarded as an unavoidable part of the life histories of many organisms, it seems that some clonal organisms that produce genetically identical modules may escape aging at the level of the genet (Gardner and Mangel, 1997). According to this concept, ramets may senesce, but the further production of new ramets may enable the genet to escape senescence. Many colonial marine invertebrates have been documented to undergo periodic phases of degeneration and regeneration. These include bryozoans (Palumbi and Jackson, 1983) and clonal species of sponges, corals, hydrozoans, the related botryllid ascidian *Botrylloides leachi* and many other ascidians (Jackson, 1985; Rinkevich *et al.,* 1996). The mechanisms regulating such processes are unknown, and clearly deserve further attention.

Is nonrandom senescence genetically programmed?

The life span of an organism can best be conceptualized as the sum of harmful changes and compensatory mechanisms that either delay or limit such changes, thus promoting longevity (Johnson *et al.,* 1999). A number of mechanisms have been described as potentially affecting aging and senescence in eukaryotes. These include damage mediated by reactive oxygen species (oxidative stress), genomic instability, telomere shortening, cell death, genetically programmed life extension, and systemic control of aging (reviewed in Jazwinski, 1996; Sohal and Weindruch, 1996; Johnson *et al.,* 1999). The relevance of such processes in the regulation of life span in *Botryllus* is unknown. However, the universality of the nonrandom senescent phenotype in *B. schlosseri* genets argues for the existence of an underlying genetic program controlling the aging process. In some aspects, nonrandom senescence of *Botryllus* genets resembles other senescence and life-span phenomena seen in some aclonal invertebrates. It is well documented that the timing of expression of life-history stages is an important factor regulating aging and senescence in animals with complex life cycles (Miller and Hadfield, 1990). For instance, the expression of a genetic program associated with aging early in the larval life of a nudibranch mollusc (Miller and Hadfield, 1990) or prior to the subcloning of a *Botryllus* colony (Rinkevich *et al.,* 1992; this study) may already set the limit for the maximal longevity of a genet. The observation that ramets often die simultaneously within the same blastogenic cycle, months following their separation, further validates this hypothesis. Recent studies have further implicated genetic elements, and their often complex interactions, in the manifestation of senescence in mammals (Haan *et al.,* 1998) and invertebrates (Orr and Sohal, 1994; Gems, 1999; Taub *et al.,* 1999). One such study documented the life spans of individual mice originating from a recombinant inbred strain. Their findings indicated that, despite a homogeneous genetic background, life spans fell into two temporal groups, revealing a locus that causes variability in the rate at which members of a population die (Haan *et al.,* 1998). In the nematode *Caenorhabditis elegans,* at least 39 genes have been demonstrated to significantly affect worm life span (Murakami and Johnson, 1998; Gems, 1999; reviewed in Johnson *et al.,* 1999; Taub *et al.,* 1999).

The role of the vascular system in programmed mortalities

An intriguing observation reported here concerns the systemic nature of vasculature constriction and that this change appears to underlie all subsequent events within senescent colonies. These findings raise the possibility that in *B. schlosseri,* nonrandom senescence could be triggered by a humoral factor that initiates the vascular anomalies and that may be transferred throughout the colony *via* blood. Our previous findings in *B. schlosseri* chimeras (Rinkevich *et al.,* 1992) indicated that the onset of senescence occurred synchronously in both partners at the time of the earliest senescent partner. These observations were completely reproduced with vascular chimeras grown in the field, but not in the majority of laboratory cohorts (Chadwick-Furman and Weissman, 1995b). The reason for the discrepancy

within laboratory chimeras is not immediately apparent, although the sample size in the study was small. These data tentatively suggest that senescence could be transmitted systemically through blood. Systemic control of the aging process has recently received experimental support in both nematodes (Apfeld and Kenyon, 1998) and mammals (Kuro-o *et al.,* 1997). According to this view, humoral factors coordinate the rate of aging in various tissues or organs (Johnson *et al.,* 1999). In mammals, a gene encoding a humoral factor termed *klotho* was recently implicated in the suppression of a wide variety of aging phenotypes (Kuro-o *et al.,* 1997). A defect in the expression of this gene in the mouse results in a phenotype that severely curtails life span, and leads to the development of aging-related pathologies. A more recent study in *C. elegans* using genetic mosaic animals for *daf-2,* a gene involved in a signal transduction pathway affecting resistance to oxidative stress and longevity, has shown that a small number of mutant cells in several sublineages were sufficient to confer enhanced longevity on the entire animal (Apfeld and Kenyon, 1998). A key finding in their study was that the ratio of mutant:wild-type *daf-2* was the most critical determinant affecting life span. These findings strongly suggest that *daf-2* may control the production of a humoral factor, and that the systemic levels of this factor determine the rate of aging in this species (Apfeld and Kenyon, 1998). The observation of a common, widespread vascular anomaly in the initial stages of nonrandom senescence in *Botryllus* lends credence to the hypothesis that aging and senescence are under systemic control. Further studies aimed at characterizing the nature and pattern of expression of this putative factor present in the vasculature of senescent colonies will be required to address this issue.

The vascular changes described herein may explain the underlying ultrastructural morphology observed. All of the visceral tissues examined revealed changes that were consistent with ischaemic cell death or oncosis, a term coined by Majno and Joris (1995). The characteristic ultrastructural changes associated with this mode of cell death, seen during nonrandom senescence in *B. schlosseri,* included cellular swelling, decrease in cytoplasmic density, organelle distension, and ultimately necrotic cell lysis, an end-point of this type of cell death (Abbott, 1983; Majno and Joris, 1995). This morphology differed from that observed during the takeover phase of the blastogenic cycle, in which apoptosis is the primary mode of cell death in the zooid viscera (Lauzon *et al.,* 1993). These subcellular features presumably corresponded to the enlarged, weakly basophilic cells seen in histological sections. On occasion, large empty spaces were also observed interspersed within the tissues of senescent zooids. These findings are interpreted as resulting from necrotic cell lysis, since no similar changes were observed in tissue sections from nonsenescent animals (Lauzon *et al.,* 1992; Rinkevich *et al.,* 1992). Since mitochondrial swelling appeared to either precede or accompany other ultrastructural changes, loss of mitochondrial function may underlie the subcellular morphology observed during nonrandom senescence. According to this scenario, a decrease in blood flow within a zooid, triggered by systemic blood vessel constriction, would limit the amount of oxygen delivered to visceral tissues. This state of ischaemia may in turn trigger a mitochondrial catastrophe, resulting in loss of ATP production. The subsequent failure of ionic pumps within the plasma membrane, a documented feature of ischaemic cell death (Majno and Joris, 1995), would in turn lead to an increase in cell permeability and ultimately to necrotic cell lysis as an end-point. Consequently, this model predicts that the subcellular changes observed during nonrandom senescence are secondary to the systemic changes occurring within the vasculature. However, the model does not provide a satisfactory explanation as to why zooids degenerate before buds do. Perhaps metabolic rate and oxygen consumption are substantially greater in filter-feeding zooids than in developing buds. Additional studies will be required to resolve this issue.

Cells with the characteristic ultrastructure of apoptosis were not directly observed during nonrandom senescence. However, some free or engulfed pyknotic cells were observed under bright-field microscopy; similarly, cells undergoing secondary necrotic lysis within phagocytes (phagolysosomes) were observed by electron microscopy. Secondary necrotic lysis is the fate of an apoptotic cell that has been engulfed by a phagocytic cell (Lauzon *et al.,* 1993). Consequently, the possibility that some apoptosis occurs prior to day 3 cannot be excluded, since electron microscopy samples were processed only at day 3 of the senescent phenotype. However, our findings are more consistent with the scenario that systemic changes, transmitted via blood, trigger ischaemic cell death with accompanying phagocytosis of dying cells. Furthermore, genetic data from other species do not support a role for apoptosis in aging and senescence. In *C. elegans,* the genetic programs underlying both processes do not overlap (reviewed in Johnson *et al.,* 1999).

We have previously shown that the vasculature also plays an important role in the clearance of dying tissues of the zooid viscera by macrophages during takeover. During this process, the hearts of regressing zooids continue pulsating until the zooids are almost completely resorbed, and thus blood flow in the dying zooid is uninterrupted throughout takeover (Lauzon *et al.,* 1992, 1993). The buds are also essential collaborators in this process, since their surgical removal in a colony markedly reduces the rate at which zooids become resorbed (Lauzon, unpubl. data). The vascular anomalies observed during nonrandom senescence could also explain why zooids are not completely resorbed during takeover. Since blood flow gradually slows down owing to the systemic constriction of all blood vessels, buds become developmentally arrested without a functional vas-

culature. Consequently, when a colony undergoing nonrandom senescence enters takeover, the parental generation of zooids cannot be completely resorbed because both vasculature and buds become incapacitated. In summary, the findings reported in this paper indicate that nonrandom senescence in *B. schlosseri* at the level of the genet exhibits a morphology and temporal sequence that is distinct from other death processes in this organism. Our observations may pave the way to understand how aging is regulated in this clonally-modular urochordate.

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