CHAPTER 6

Hunting for Pathogens: Ancient DNA and the Historical Record

S. Marciniak
Department of Anthropology, McMaster University, Hamilton, ON, Canada

The bioarchaeological investigation of disease benefits from an interdisciplinary approach because the disease process itself is multifaceted; its expression is responsive to the environment, and is influenced by human activity. Ancient DNA is a powerful research tool, capable of addressing diverse questions surrounding the origin, distribution, susceptibility, or evolutionary changes of a pathogen and the disease it may cause (eg, Devault et al., 2014a; Spigelman et al., 2015; Wagner et al., 2014). Upon integration with other lines of evidence such as ancient literary texts, archaeological data, or skeletal analyses, the experience of disease in antiquity may be illuminated.

The integration of ancient DNA analysis in a bioarchaeological study typically relies on selecting skeletal samples associated with presumed pathogens. Disease may be identified by diagnostic skeletal changes (eg, *facies leprosa* indicative of leprosy; Andersen and Manchester, 1992), catastrophic assemblages from epidemics or plagues connected to an infectious agent (eg, *Yersinia pestis* and the Black Death; Bos et al., 2011; Drancourt et al., 1998), or literary evidence describing symptomology in the wake of mass mortality (eg, Plague of Athens; Page, 1953). Such exclusive association is rare in the archaeological record due to the Osteological Paradox: (1) few infectious diseases induce skeletal responses, as only 5–20% of individuals manifest pathological changes (Ortner, 2008; Roberts and Manchester, 2005; Steinbock, 1976); (2) chronic or long-term infections (eg, tuberculosis, leprosy) are predominant, whereas acute infections of rapid mortality or spontaneous recovery (eg, bubonic plague, malaria) leave no skeletal traces (Roberts and Manchester, 2005; Steinbock, 1976); and (3) susceptibility to disease (heterogeneity and frailty)

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means that there is individual variability in responses to infection (Wood et al., 1992; Wright and Yoder, 2003).

This chapter presents a scenario exploring infectious diseases in three Imperial Roman necropoleis (ancient cemeteries) (Italy, 1st–4th century CE), utilizing disparate datasets: ancient DNA analysis of skeletal remains, complemented by literary and archaeological evidence.

6.1 EXPLORING DISEASE IN IMPERIAL ROMAN NECROPOLEIS

The cemeteries of Vagnari (2nd–4th century CE; see Prowse et al., 2010; Small, 2011, 2014), Velia (1st–2nd century CE; see Craig et al., 2009; Crowe et al., 2010; Fiammenghi, 2003), and Isola Sacra (associated with Portus Romae) (1st–3rd century CE; see Bondioli et al., 1995; Prowse et al., 2005, 2007) are geographically disparate necropoleis in Italy. The individuals buried at Vagnari likely represent a workforce associated with a rural inland estate far from urban centers (Prowse et al., 2014; Small, 2011, 2014), while Velia and Portus Romae were important port cities and maritime trading centers, closely connected to the Roman capital (Craig et al., 2009; Keay and Paroli, 2011; Prowse et al., 2007). Similar to other skeletal assemblages indicating health stressors across Roman Italy (Cucina et al., 2006; Eckardt, 2010; Facchini et al., 2004; Killgrove, 2010), there is skeletal evidence of nonspecific stressors (eg, linear enamel hypoplasias, cribra orbitalia, porotic hyperostosis) at Vagnari (Prowse et al., 2014), Isola Sacra (Gowland and Garnsey, 2010), and Velia (Beauchesne, 2012). In such contexts, ancient DNA facilitates the identification of disease-associated pathogens since it is difficult to diagnose specific diseases based solely upon skeletal changes.

To comprehensively investigate morbidity and mortality within these Imperial period assemblages, it is necessary to apply an approach mentioned by Killgrove (2014) that systematically considers multiple factors associated with a dynamic disease-scape, such as malnutrition, sanitation and hygiene, social status, urbanization, and infrastructure as well as cocirculating and coinfecting pathogens. The objectives of the necropoleis project outlined in this chapter are twofold: (1) assess candidate pathogens through metagenomic "shotgun" sequencing of the entire microbial DNA as a means to infer pathogen presence; and (2) contextualize the molecular data using archaeological evidence related to disease ecology at each site and evaluate the degree to which

selected ancient literary texts enable inferences of prospective diseases beyond the Roman capital to other parts of Roman Italy. The goal is to explore factors mitigating or proliferating disease in diverse assemblages of the Imperial Roman period using a framework that emphasizes the multifactorial pathways of disease.

6.2 CHARACTERIZING ANCIENT PATHOGENS

6.2.1 Ancient DNA and Pathogen Detection

For the Roman necropoleis, a single tooth was selected from 20 adult individuals in each necropolis (for a total of 60 teeth) as part of a large-scale screening approach to maximize the detection of pathogens alongside the range of environmental, commensal, and contaminant microorganisms within individual metagenomes. Shotgun sequencing was applied as a screening method to evaluate whether human pathogens, based on Roman historical and bioarchaeological evidence, are identifiable in the metagenomic datasets generated from the Roman necropoleis samples, rather than as a tool for the definitive identification of pathogens.

In integrating ancient DNA in bioarchaeological studies, there are a number of issues that merit consideration. Primarily, ancient DNA exhibits signatures of molecular and chemical degradation alongside idiosyncratic preservation (intra- and inter-specimen variability), resulting in an abundance of damaged short DNA fragments (on average 30–60 base pairs or bp) compared to modern biological DNA (see reviews by Hofreiter et al., 2014; Molak and Ho, 2011). These factors directly affect the complexity of DNA constituents, which predominantly contain environmental (eg, soil, sediment, or macroorganisms, such as plants or fungi), modern contaminant, and non-target DNA, with a low endogenous content (eg, 0–5%) (Burbano et al., 2010; Carpenter et al., 2013; Pääbo et al., 2004). There are notable exceptions, however, where well-preserved genomic data are retrievable, such as permafrost specimens (see Bellemain et al., 2013; Legendre et al., 2014). The challenge is that the minute pathogen DNA fraction in human bones and teeth is embedded within the entirety of a dynamic DNA "pool," and this poses a significant obstacle to resolving historical pathogen presence.

When applying ancient DNA methodologies in a context such as the Roman necropoleis, where disease-associated pathogens are

unknown, it is critical to consider the appropriate specimen to enable broad-spectrum detection of blood-borne pathogens. Teeth are considered optimum substrates—particularly subsamples of the root or recovering dental pulp—due to substantial vascularization and increased protection from molecular degradation, whereas bone is a less ideal substrate, as it can absorb significant amounts of contaminant DNA from the burial environment (Adler et al., 2011; Drancourt et al., 1998; Higgins and Austin, 2013).

Metagenomic "shotgun" sequencing or sequencing without the selection of targets (ie, loci, genes, genomes) is useful when there is no knowledge of a presumed disease and in consideration of the confounding host and environmental factors (eg, host susceptibility, heterogeneity of risk) contributing to the expression of disease (Devault et al., 2014b; Orlando et al., 2015; Warinner et al., 2015). Shotgun sequencing demonstrates success in ancient DNA research, such as Kay et al. (2014) obtaining 6.5-fold coverage of a *Brucella melitensis* genome from the calcified nodule of a medieval specimen. The approach is the least biased, compared to amplicon sequencing (16S rRNA genes) or targeted capture strategies (these require a presumed pathogen), and is the most comprehensive means of characterizing the entire microbial DNA content of a sample (Devault et al., 2014b; Warinner et al., 2015; Whatmore, 2014). A caveat with shotgun sequencing is that low abundant human pathogens are often undetectable within these substantial metagenomic datasets (eg, comprising less than 0.08% of sequence reads in Devault et al., 2014b) and it remains cost-prohibitive to deeply sequence samples for diagnostic pathogen identification (defined as greater than 30× for meaningful genome coverage).

6.2.2 Metagenomic Pathogen Screening From the Necropoleis

As noted by Scheidel (2009), the uncertainties surrounding the knowledge of health and disease in ancient Rome enables diverse scenarios to be constructed from multiple evidentiary sources. In this sense, the framework applied to prioritize candidate disease-associated pathogens in Velia, Vagnari, and Portus Romae relies on historical reasoning and drawing contextually relevant factors from the disparate sources.

It is beyond the scope of this chapter to comprehensively discuss the bioinformatic processing of the Roman sequence data and the specific metagenomic results (eg, microbial diversity and taxa abundance, data

authentication). Samples were processed at the McMaster Ancient DNA Centre (Hamilton, ON, Canada), with physically separated ancient DNA and modern laboratory facilities. DNA was extracted using a protocol modified from Schwarz et al. (2009) and libraries suitable for sequencing on the Illumina HiSeq 1500 platform (75 bp paired-end read chemistry) (Illumina, San Diego, CA, United States) were prepared with modifications in previously published protocols (Kircher et al., 2012; Meyer and Kircher, 2010). Raw sequence reads were trimmed to remove residual adaptors and then merged using SeqPrep (St. John, 2011).

Briefly, 180,000 to 8,000,000 raw (unprocessed) DNA reads were generated across the Roman samples and the processed metagenomic datasets varied from 500,000 to 4,000,000 sequence reads across the 60 Roman libraries (one for each tooth sample). Negative controls (ie, extraction blanks) were processed alongside the Roman libraries and demonstrate low abundance bacterial sequences (less than 300 reads).

To establish that the retrieved ancient DNA is authentic, protocols are proposed to prevent and detect contamination (eg, Cooper and Poinar, 2000), including separate ancient and modern DNA laboratories, unique nucleotide damage patterns associated with ancient specimens (cytosine deamination near the 5′ ends of fragments), and appropriate molecular behavior (the degradation of DNA into an abundance of short fragments compared to few long fragments) (see Briggs et al., 2009; Brotherton et al., 2007; Lindahl, 1993). Bioinformatic estimates of contamination (eg, Ginolhac et al., 2011; Skoglund et al., 2014) require mapping the sequences to a reference (eg, a specific pathogen genome or genes), which is not always possible with metagenomic data (as mentioned by Warinner et al., 2015). This is observed in the Roman data discussed here, with a low number of reads (and subsequent low coverage) when mapping to candidate human pathogen genes.

The onus is on the researcher to critically analyze the project design and incorporate relevant criteria to evaluate the validity of the sequence data (Gilbert et al., 2005). Accordingly, the proportion of reads from the Roman samples mapping (matching) to the human mitochondrial genome (revised Cambridge Reference Sequence) (as a proxy for inferring authenticity) varied from 0.003% to 1.2%, and the sequences demonstrate signatures of authentic and

highly degraded ancient DNA as evidenced by higher rates of deamination (C to T and G to A transitions near the 5′ and 3′ ends of fragments, respectively), read length distributions indicating the predominance of short DNA sequences (24–60 bp), and characteristic fragmentation patterns.

The Roman shotgun data were processed to identify taxonomic assignments by comparison to a nucleotide sequence database (blastn-megablast, v.2.2.29) (Altschul et al., 1990). Each BLAST (Basic Local Alignment Search Tool) output was parsed using MEGAN4 (MEtaGenome ANalyzer, v.4.70.4) in order to assign a taxon (Huson et al., 2007). The results of the MEGAN4 output demonstrate extensive metagenomic diversity, capable of Family- to Species-level characterization, with a range of 3–30% of reads taxonomically identifiable across all samples. The Bacteria taxon is the most predominant in the number of assigned reads, followed by the Eukaryota (eg, Fungi, Viridiplantae) and Hominoidea taxa. Comparisons of the taxonomic distributions across the samples depended on the resolution of the data (ie, number of reads taxonomically identified to the Genus or Species level, number of unassigned reads), and ranges from soil organisms characteristic of salt/marsh environments (eg, *Desulfovibrio* spp., *Aeromonas* spp.), to organisms of the human microbiome (eg, skin and gut flora, such as *Propionibacterium*) and pathogenic components (eg, periodontal disease agents, such as *Tannerella forsythia*). Human-only pathogens are present in low abundance in the Roman metagenomic dataset, varying from 0.03% to 3% (or 0.9% on average) of taxonomically identifiable sequence reads. The presumptive pathogens identified and selected as candidates for further analysis included: *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Clostridium botulinum*, and *Clostridium tetani*.

There are challenges in using taxonomic assignments as a means to identify candidate pathogens from the necropoleis of Velia, Vagnari, and Isola Sacra. Methodologically, this confidence threshold relates to a key parameter of MEGAN functionality, the ability to assign short reads (less than 50 bp) to a taxonomic level (Huson et al., 2007). The low number of human pathogen taxa observed with the Roman metagenomic data is partly attributed to the short read lengths, ultimately producing fewer taxa assignments, thereby underpredicting the number of reads associated with a taxa and actual metagenomic

content (Huson et al., 2007). MEGAN's computational approach emphasizes sensitivity, but the conservative measure of taxon identification limits the use of the Roman metagenomic dataset to definitively include or exclude the presence of specific disease-associated pathogens. This means that a particular taxon assignment is not irrefutable evidence of a target's presence, nor is the absence of pathogen DNA certain when not identified taxonomically. The degree to which any pathogen is identified in the metagenomic dataset requires examination of the reads that are the basis of its taxonomic assignment. Alignments and mapping to the presumed pathogen genome (or genes) are also important to identify the particular genomic regions used to "call" the taxonomic identification.

In this sense, although pathogen candidates are identifiable from the shotgun data, it is unknowable which pathogens have been undetected or underrepresented with this molecular strategy. The sequence depth of one million reads per sample is insufficient to fully characterize the necropoleis samples' genomic composition; however, without previous molecular work on these ancient Roman samples, shotgun sequencing provides a preliminary assessment of predominant taxa abundance and diversity, with indications of disease-associated pathogens present to later target via hybridization capture (ie, the sequestration of DNA via known sequence probes or "baits"). To address the challenges of implementing a shotgun sequencing strategy using previously uncharacterized ancient samples to evaluate pathogen presence, it is critical to further refine the nosological context by identifying contextually relevant diseases presumed to be present in ancient Rome to evaluate applicability within the necropoleis under study, and establishing the epidemiological environment at the sites.

6.2.3 Integrating Metagenomic Data With the Historical Record

6.2.3.1 Literary Texts and Disease-Associated Pathogens

The use of ancient written sources presents a unique set of challenges in reconstructing disease presence in the past, since texts represent a specific context of experiencing and understanding disease (Leven, 2004; Rosenberg, 1992). From the available range of ancient Greek and Roman literary evidence, scholars infer circulating diseases within the Roman world to include tuberculosis, typhoid fever, malaria, leprosy, gastroenteritis, cholera, dysentery, syphilis, herpes, and brucellosis (Grmek, 1989; Nutton, 2004; Sallares, 2002; Scheidel, 2003, 2009);

however, it is critical to recognize the relationship between the humoral theory of the Hippocratic tradition during this time and the practice of retrospective diagnoses.

Within humoral theory, there were no unique causal explanations for disease and the focus was on discovering the underlying humoral imbalance in the individual that produced the observed illness (Grmek, 1989; Nutton, 2004). The translation of these ancient descriptions into diagnosed "named diseases" inadvertently imposes contemporary knowledge about diseases upon the past (Grmek, 1989; Mitchell, 2011; Nutton, 2004). For example, the use of "lepra" (scaly, thickened skin) in the Hippocratic tradition indicates a variety of benign dermatological conditions, such as psoriasis or eczema, not solely *Mycobacterium leprae*, as it is understood today (Nutton, 2004). This means a variety of conditions or diseases may have been associated with these descriptions; accordingly, it was necessary to integrate an approach that did not focus on establishing "named diseases" from these texts, but evaluated the picture of disease expression (or a syndromic approach) proposed by Muramoto (2014) by applying contemporary epidemiological concepts (ie, factors proliferating or mitigating disease).

Within the retrospective interpretation of diseases, it is also critical to be aware of the particularities related to the Roman context. The literary record itself is highly fragmentary with a scarcity of documentation on chronic diseases and epidemic outbreaks during the Late Republican and Imperial periods as noted by Scheidel (2009) where only 15 epidemics were recorded from the 2nd century BCE to the 8th century CE. The practice of ancient authors copying from earlier texts without acknowledging predecessors also complicates assessment of whether descriptions were developed with firsthand knowledge of the disease (Grmek, 1989; Mitchell, 2011; Nutton, 2004). These factors necessitate emphasis on the Roman context of experiencing and understanding disease as shown by the literary evidence. Due to an absence of specific written evidence (eg, medical texts, personal accounts) for the necropoleis of Vagnari, Velia, and Isola Sacra, select ancient texts are used to draw inferences of the disease experience at a given time (defined as nosology, by Grmek, 1989) in the broad context of the Imperial period. Translated and original versions of ancient Greek and Roman medical texts associated with the Late Republic to Imperial

periods used in this study included: (1) works of the Hippocratic Corpus (see Adams, 1891; Jouanna, 1999); (2) writings of the physician Galen (see Kühn, 1825); (3) Pliny the Elder (see Rackham, 1938); and (4) Celsus (see Collier, 1831).

In this sense the picture of disease is framed by the characterization of the Roman capital as similar to premodern urban societies (eg, preindustrial London) or a contemporary third world country (Dyson, 2010; Nutton, 2004; Scheidel, 2009), in order to facilitate the presumptive association of contextually relevant diseases, such as chronic infections (eg, tuberculosis, leprosy, syphilis), acute diseases (eg, smallpox, cholera), and opportunistic infections (eg, gastroenteritis attributed to *Salmonella* spp., *Staphylococcus* spp.) (eg, Grmek, 1989; Nutton, 2004; Scheidel, 2003, 2009). The inferences of infectious and parasitic diseases as the predominant agents in the pathogen pool of the Roman Empire are viewed as outcomes of a densely urbanized context, complicated by poor nutrition, crowded conditions, "unsanitary" practices (eg, improper corpse disposal, ineffective sewage systems), and environmental influences (eg, proximity to the Tiber River, flood frequency) (Dyson, 2010; Gowland and Garnsey, 2010; Scheidel, 2003, 2009). Descriptions drawn from not only medical texts, but also Roman practices of food preparation, animal husbandry, farming, or other daily activities may further outline the scope of disease-associated pathogens (most applicable for the rural estate of Vagnari), rather than those solely associated with preurban landscapes.

However, a complication with integrating the Roman metagenomic data in consideration of the historical record is that the geographic distribution of diseases is unknown within the Empire. References within the literary record are also restricted to specific locations, such as the Roman capital; while the Hippocratic Corpus is geographically confined to Thessaly and northern Greece, documenting selected cases of interest (Jouanna, 1999). This is a limiting factor in attempting to explore the seasonality or spatial range of morbidity or mortality associated with disease, particularly for the sites of Velia, Vagnari, and Portus Romae. Hippocrates' "Aphorisms" (III.XX–XXIII) (Adams, 1891) indicates specific diseases were exacerbated at particular times, such as continued, ardent, and tertian fevers in the summer or irregular fevers, dropsy, and phthisis in autumn. Inferences may be drawn from this text regarding seasonality of symptoms associated with descriptions

of diseases; however, the coastal locations of Velia and Portus Romae, and the rural location of Vagnari further create a dynamic epidemiological environment due to diverse pathogen reservoirs and related human risk of exposure. Accordingly, the explanatory framework provides a picture of presumed diseases associated with life in the Roman Empire, but it is critical to consider the context from which these interpretations are based, as the conditions creating the pathogen pool in the Roman capital are not equivalent to the forces contributing to the pathogen burden at Vagnari, Velia, and Portus Romae.

Broad-based generalizations about the disease-scape in ancient Rome drawn from the literary record provide a picture from which to make inferences relating to the populations of Velia, Portus Romae, and Vagnari, although it is recognized that disease distribution was likely extremely heterogeneous. Going beyond generalized conclusions relating to the presence of diseases associated with a metropolitan environment is challenging for the rural and suburban Roman Italian populations. However, building the nosological context requires additional data from archaeological evidence related to disease ecology and is beneficial in refining the influences on pathogen exposure and disease expression.

6.2.3.2 Archaeological Data and the Disease-Scape

Archaeological evidence for human-modified landscapes and disease ecology is evaluated to frame potential exposure to pathogens within the necropoleis studied. The application of ecosocial epidemiological theory (Krieger, 2011) as part of an ecosystem approach to health (Waltner-Toews, 2001) creates a framework for the identification of factors potentially proliferating or limiting disease within Velia, Vagnari, and Portus Romae. Krieger's (2011, p. 215) concept of the "lived experience of disease" also guided the identification of ecological factors potentially affecting the retrospective burden of disease in these populations. Drawing from these frameworks, critical ecological components affecting the proliferation of disease(s) and causative agents were evaluated as the following: climate; landscape (type of soil or sediment, types of vegetation); geomorphology (topography changes, coastline formations); water bodies (lagoons, swamps, lakes); and extreme events (flooding, periods of aridity). This evidence was then applied in inferring disease ecology and the sustainability of pathogen transmission or survival at the local and regional scales.

The manifestation of disease is further structured within sociocultural and political realms (Krieger, 2008) and, accordingly, the evaluation of human–environment interactions considered the impact of anthropogenic processes (eg, urbanization, agricultural practices, construction activities, trade, and migration) as active modifiers of disease ecology and the resultant disease burden (Krieger, 2011). In evaluating human agency as broadly modifying the pathogen pool within ancient Rome and the necropoleis in particular, a number of inferences were drawn from current epidemiological knowledge regarding disease transmission and sustainability in preurban landscapes (eg, population density, public infrastructure, human mobility). Integrating aspects of the paleoenvironment alongside evidence of human activities facilitates the reconstruction of a snapshot of the dynamic disease pool associated with the epidemiological environments of Velia, Vagnari, and Portus Romae at a given moment in historical time.

In using paleoenvironmental data from Vagnari, Velia, and Portus Romae, as well as the Mediterranean region to draw inferences on the factors proliferating or mitigating potential disease-associated pathogens, there is dynamic potential in each environment to sustain pathogens; however, the ability of local ecosystems to modulate pathogen presence and exposure is not fully discernible solely through the archaeological record. Mediterranean pollen records indicate a warm and dry climate, typifying the current subtropical climate and landscape of indented coastlines, valleys, forests (coastal and woodland), and shrubland (Grove and Rackham, 2001; Sadori et al., 2011). However, it is only possible to broadly interpret the subtropical Roman Italian environment as *capable* of supporting vector-borne and parasitic diseases typically associated with such subtropical regions (WHO, 2015). Although the available paleoenvironmental evidence from Velia, Portus Romae, and Vagnari is variable, there remains the potential to evaluate the known and unknown factors contributing to local disease ecology.

At the scale of Vagnari, Velia, and Portus Romae, paleoenvironmental data frame the conditions of the ancient environments within which individuals survived and thrived. The geographic distribution of pathogens within these ancient environments is affected by the landscape where the coastal geomorphology of Velia

(cliffs, dunes, marshes, and bays; Amato et al., 2010) and Portus Romae (marshes, hills, dunes, and coastal woodlands; Di Rita et al., 2010; Keay and Paroli, 2011) contrast to inland Vagnari (lowland hills, river valley; Small, 2011, 2014), thereby highlighting the potential for heterogeneous distribution of pathogen reservoirs. For example, within Portus Romae the gradual and seasonal desiccation of a lagoon and marsh has consequences for vector colonization of surface pools (Di Rita et al., 2010; Keay and Paroli, 2011). The effect of extreme climatic events, such as flooding, affects the capability of public infrastructure and exacerbates infectious diseases (Bissell, 1983; Ivers and Ryan, 2006). For example, Portus Romae is in close proximity to the Tiber River with its record of recurrent floods (5th century BCE to 4th century CE) (Aldrete, 2007; Keay and Paroli, 2011), while the stratigraphy at Velia indicates flood events through sedimentation of alluvial deposits (Amato et al., 2010; Ruello, 2008). Although the paleoenvironmental evidence is limited among the sites, each merits evaluation regarding local disease ecology and the potential impact on the distribution of disease-associated pathogens. The integration of metagenomic data identifying candidate pathogens for each site, alongside explanatory frameworks of disease in the Imperial period, provides a means to contextualize the manifestation of these forces in the specific paleoenvironments of Velia, Vagnari, and Portus Romae.

Proxies of the causal pathways behind disease exposure and susceptibility (eg, population density, infrastructure, scale of urbanization, biocultural environment), as drawn from the Roman historical record and contemporary epidemiological knowledge, are integrated as a means to infer the influence of human activity on disease distribution at each site. The absence of demographic information for Velia, Portus Romae, and Vagnari limits inferences regarding transmission and survival of infectious diseases in suburban and rural contexts. Infrastructure is a critical determinant of population health, particularly the efficacy of Roman aqueducts and sewage systems in providing clean water while removing waste. The overflow from public basins and fountains is argued to contribute to surface cesspools of gastroenteritis, dysentery, cholera, and helminth infections (Scheidel, 2003, 2009; Scobie, 1986). For example, the proximity of residences to aqueducts in Portus Romae (Keay and Paroli, 2011), the terrace system in Velia (Amato et al., 2010), and the water-filled ravine that ran through Vagnari (Small, 2011) raise the issue of the potential for

seepage or surface pools alongside flat stretches of land (Keiser et al., 2005). Similarly, the construction of buildings or residences (single or multistorey) may indicate the number of inhabitants, which relates to the potential for the communicability of disease. The distribution of such residential complexes, industrial and ceremonial buildings further contributes to varied patterns of disease.

Economic and political activities as outgrowths of urbanization during the Imperial period are significant in assessing the potential influence on pathogen burden. Maritime trade and immigration are capable of introducing novel pathogens into the population (Killgrove, 2014; Prowse et al., 2007; Scheidel, 2009), which relate to the sustainability of pathogen pools in the port cities of Velia and Portus Romae. The identification of nonlocal individuals at the sites of Vagnari (Prowse et al., 2010) and Portus Romae (Prowse et al., 2007) through isotopic or ancient DNA analysis is critical to address the distribution of disease among the inhabitants. The broad range of factors associated with servicing a growing Empire, such as irrigation canals as part of agricultural practices, road-building to expand connections among cities, and deforestation to harvest wood/timber potentially affect the frequency of human encounters with pathogen reservoirs (Sutherst, 2004; Yasuoka and Levins, 2007). For example, the Via Portuensis (linking Rome and Portus Romae) was constructed with small stone layers potentially proliferating the longevity of standing water (Keay and Paroli, 2011). The construction, frequency, and manner of maintenance for public infrastructure directly influence the efficacy of these systems in evaluating factors mitigating or proliferating disease within respective populations.

6.3 MULTIFACETED EVALUATION OF DISEASE-ASSOCIATED PATHOGENS

There is an inherent range of variability in inferring pathogen presence in Roman Italy with the selected data sources; however, the objective is to systematically identify contextually relevant variables to conceptualize the diverse causal pathways of disease occurrence as a means to target candidate pathogens for further analysis (eg, hybridization capture). Reconstructing the paleoepidemiological context through disease ecology and human–environment interactions using proxy evidence is necessarily limited by the availability of such data and their

specificity to the sites studied as well as the degree to which one can infer the potential for pathogen exposure without drawing tenuous interpretations. For example, if parasitic infections are presumed to be part of the disease pool in the Roman Empire, and shotgun sequencing does not detect these pathogens within the coastal and rural skeletal samples within this study, their absence means it is not a focus of further analysis due to a reduced likelihood of success in targeting these pathogens with a molecular capture strategy. The caveat is that although the pathogen is undetected, it does not mean it was not present, thereby requiring cautious interpretation of the metagenomic evidence. Integrating archaeological and literary evidence can facilitate whether these pathogens are suitable for targeting with downstream processing; however, the endeavor is of high risk (diminishing returns). Similarly, the "unexpected" detection of a pathogen is not uncommon, as the historical Greek and Roman literary sources cannot be directly applied to the necropoleis in this study, and archaeological data provide only broad information on the potential (not absolute) hypothesized range of pathogens that may have been present. However, these integrated datasets do provide a framework for hypothesizing the pathogens that may have been present and are practical to pursue in a purposeful manner. The ultimate goal of integrating molecular, archaeological, and literary sources toward a framework of decision-making is to evaluate a variety of separate but interconnected factors that combine to create the ancient disease-scape (Fig. 6.1).

6.4 TOWARD INTERDISCIPLINARY ANCIENT DNA AND PATHOGEN INVESTIGATION STRATEGIES

The perspectives provided by each type of evidence: ancient DNA, written texts, and archaeological data represent one facet of exploring disease in antiquity. Ancient DNA data are a contemporary biomedical picture of disease, whereas the written and archaeological sources are conceptualizations of a moment in historical time.

The disease ecology at Vagnari, Velia, and Portus Romae is amenable to inferences of factors mitigating or proliferating pathogen exposure, although only a limited range of information is available (eg, climate, extreme weather events) and the resilience of local

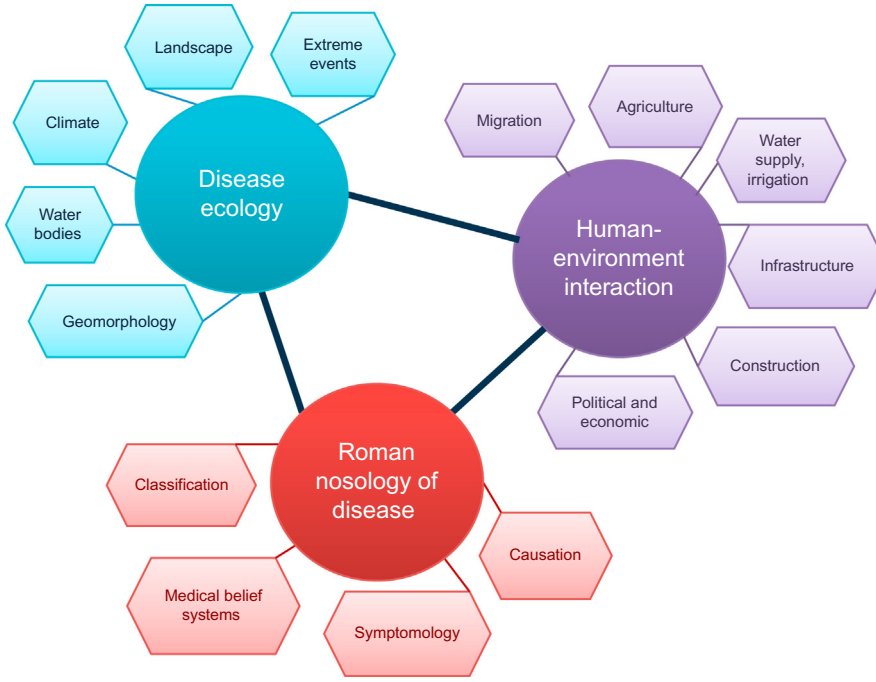

Figure 6.1 Framework integrating aspects of disease ecology, Roman nosology, and the human–environment interaction to situate molecular data in exploring historical pathogen presence and inferring the experience of disease.

ecosystems to such factors is unknowable. Similarly, humans modify pathways of pathogen exposure by interacting with the environment in diverse ways, whether on the scale of urbanization activities (eg, construction) or day-to-day life (eg, farming, person-to-person contact). Although the context of human activities may be established at the site-level, the consequences of such activities on the long- or short-term composition of the pathogen pool can only be effectively reconstructed by integrating this information with ancient DNA evidence, particularly because skeletal remains often do not display clear evidence of infectious diseases. With metagenomic data, identifying contextually relevant disease-associated pathogens from a sample with high proportions of exogenous DNA presents a formidable, but not insurmountable challenge, requiring a conservative approach toward establishing pathogen presence. It is only through the systematic integration of various evidentiary sources that the complexity of pathogen presence and inferences of the disease experience in the past can begin to be investigated.

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