SEX IDENTIFICATION OF HUMAN REMAINS FROM AN IRISH MEDIEVAL POPULATION USING BIOMOLECULAR METHODS

Sheila Tierney, B.Sc., PhD
School of Science, Institute of Technology Sligo, Ireland.
Dr Jeremy Bird, B.Sc., M.Sc., PhD
School of Science, Institute of Technology Sligo, Ireland.

Abstract

The excavation of a medieval cemetery in County Donegal has allowed the recovery of one of the largest assemblages of human remains from a burial ground in Ireland to date. In order to enhance the interpretation of the assemblage and give a more complete picture of the Ballyhanna community as a whole, a sample of the juvenile population from Ballyhanna were sexed using DNA based techniques. Sex identification of human remains is generally assigned using skeletal morphology or on some occasions using associated grave goods. When an assemblage contains immature or fragmentary material, an alternative and reliable means of sexing these individuals is required. In this research project the reproducibility of two PCR based sexing methods were evaluated on 38 adults of known sex to determine the accuracy of the methodology for sexing individuals from the Ballyhanna assemblage. Using real time PCR and STR profiling, a reliable sexing system was developed for adult remains which was validated against osteoarchaeological sexing. The reproducibility of the results from the amplified samples meant that the methodologies were valid and could be used to sex juveniles from the assemblage. The molecular sexing results from nineteen juveniles sampled determined that four of the juvenile individuals were males, 10 were probable males, one was a probable female and four were inconclusive. The results from this study, although they may not be fully representative of the entire juvenile population excavated from this cemetery, indicate higher levels of juvenile male mortality than female.

Keywords: Ancient DNA, Human, Molecular, Sex determination, Medieval

Introduction

Sex identification of human remains is generally assigned using morphological and morphometric analyses or occasionally using associated grave goods. Juvenile individuals exhibit little sexual dimorphism in their skeletons and thus assigning sex to such skeletal remains is difficult. Where an assemblage contains a high number of ambiguous remains such as immature or fragmentary material, an alternative and reliable means of sexing individuals is therefore required. A number of attempts have been made to develop reliable osteological methods for diagnosing sex in juvenile material (Weaver, 1980; Holcomb & Konigsberg, 1995, Schutkowski, 1993; Cardoso, 2008) although reliable sex determination cannot be established for immature individuals using standard osteological methodologies (Brickley, 2004). Molecular sexing techniques on the other hand, have proven to be an alternative method (Cappellini et al., 2004; De La Cruz et al., 2008; Daskalaki et al., 2011).

Following the discovery of a medieval cemetery in the Ballyhanna, Co. Donegal, Ireland, in 2004, 1296 human skeletal remains were recovered comprising 869 adults and 427 non-adults. The period of burial at Ballyhanna dates from the late seventh centuryuntil

the 17th century (MacDonagh, 2012). The substantial number of non-adult remains recovered could provide insight into how children lived and died in medieval Ireland. In order to enhance the interpretation of the assemblage and give a more complete picture of the Ballyhanna community as a whole, a sample of the juvenile population from Ballyhanna were sexed using DNA based techniques so that the mortality ratio of the male and female non-adult individuals could be assessed.

The most widely used DNA based methods for sex identification targets the amelogenin gene (AMEL) which is found on the human X and Y chromosomes and can be used to distinguish between males and females due to differences between sequences on the two chromosomes (Sullivan et al., 1993; Mannucci et al., 1994).

The second method selected utilised STR analysis . STR analysis is a highly effective genetic typing method, as the number of repeats in STR markers is highly variable among individuals (Butler, 2005). The application of STR profiling in ancient DNA analysis enables reliable identification of inconsistencies between samples which have been independently replicated in two centres of analysis. STR profiling is therefore a dependable and useful approach for indicating authenticity of ancient DNA extracts (Bramanti et al., 2003). The narrow size range of the STR alleles makes them especially suitable for typing aDNA samples (Butler, 2005). Incorporating the amelogenin assay into a multiplex STR typing system enables co-amplification of the X Y homologous amelogenin gene with a number of polymorphic STRs in one reaction, which in turn allows for the sex and STR genetic fingerprint of an individual to be simultaneously obtained. Therefore not only can the sex of an individual be identified but it can be determined if the amplified DNA is endogenous to the ancient specimen or from contemporary contaminants.

Materials and Methods

Before using either of the biomolecular tools for DNA based sexing we verified that the methods were accurate and reliable by firstly validating them on morphologically sexed adult skeletons from the assemblage. By testing our methods on adults of known sex, we were able to ensure that a reliable and unambiguous method was developed for determining the sex of juveniles from the assemblage. The authors adhered to the criteria of authenticity published by Cooper & Poinar, (2000) and Pääbo et al., (2004) where appropriate for this study.

2.1Preparation of samples for DNA Extraction

Thirty eight adults and nineteen juveniles were selected randomly from the collection for analysis. Teeth which had intact crowns with minimal cracks were selected while badly eroded teeth were avoided. Teeth with poorly preserved or broken tips were also rejected as it was observed that teeth with intact root systems yielded improved profiles. When available a second or third tooth of good quality was also chosen from each skeleton for confirmation of DNA results. A reproduction of each tooth selected for aDNA analysis was made by creating a mould using dental prosthetic material (Heraeus). Each tooth was cleaned using sterile scalpels and 10%sodiu hypochlorite, before being incubation in 7.5ml of decontamination solution (Cline et al.,2003) comprising 1% SDS, 25mM EDTA, 20mg/ml Proteinase K and deionised water for 1 hour. The tooth was UV irradiated for one hour and left to dry before being ground into a fine powder using a SPEX SamplePrep cryogenic mill (SPEX). One hundred mg of tooth powder was then aseptically dispensed into two DNA free microcentrifuge eppendorfs (Sigma- Aldrich) and used for extraction. The remaining sample powder was stored at - 20ºC.

2.2 DNA Extractions

In order to prevent contamination from modern and amplified DNA, all ancient DNA extractions and pre PCR set up were conducted in a separate laboratory dedicated exclusively

to ancient DNA work (O' Rourke et al.,2000). No modern DNA analysis or PCR amplifications had been conducted in this laboratory previously (Yang & Watt, 2005). Personnel entering the pre PCR laboratory were only to do so if they had not worked in the post PCR laboratory on that same day and when wearing the correct personal protective clothing. Before any work was carried out in the ancient DNA laboratory,all surfaces and equipment were thoroughly decontaminated with 10% Sodium Hypochlorite. Surfaces, equipment and reagents used in the extraction process were also exposed to ultraviolet light for a minimum of 1 hour to degrade modern DNA template residues (Yang & Watt, 2005). All consumables used were DNA free or of a high sterile standard.

After comparing the quantitative yields from a number of extraction techniques, the DNA extraction method chosen for the Ballyhanna samples was the rapid column based silica extraction method (Rohland et al., 2010). This method was selected as it maximised DNA yields from the Ballyhanna skeletons while at the same time was cost effective and reliable. All extractions from each tooth sampled, including the extraction blank, were performed in duplicate (sample A and sample B).

2.3 Quantitative PCR

The Quantifiler Duo DNA quantification assay (Applied Biosystems) was used for a dual purpose in this study; firstly to quantify the amount of amplifiable human DNA and human male genomic DNA in each extract for further analysis such as STR profiling and secondly as a preliminary sexing assay. The Quantifiler Duo assay amplifies short target sequences thus making it ideal for detecting degraded ancient DNA. From each tooth sampled, at least two PCR amplifications were conducted from the A and B extracts. Two µlof aDNA extract was added to a 25 µl reaction and was amplified on the ABI 7500 RT-PCR system using the optimised reaction conditions recommended by the manufacturer. At least two non template controls were amplified with each set of samples to monitor for contamination.

2.4 Short Tandem Repeat (STR) Profiling

The PowerPlex® S5 System (Promega) which detects four STR loci (D18S51, D8S1179, TH01 and FGA) and the Amelogenin locus was used to generate the STR profile and identify the sex of each individual sampled in this study. The quantity of DNA in each extract, obtained using the Quantifiler Duo assay, was used to determine the amount of sample to be added to the STR reaction. The optimal amount to be added was between 0.25-0.5 ng of template DNA. In situations where the quantity of aDNA was less than this, the maximum amount of extract which could be added to the reaction (17.5 µl) was aliquoted. For the negative amplification control, nuclease free water (Promega) was added to the reaction tube instead of template DNA. The cycle number was increased from the recommended 30 cycles to 33 cycles to increase the sensitivity when using low amounts of template (Gill et al., 2000). PCR amplifications were performed according to the recommended protocol for the Applied Biosystems 2720 thermal cycler. The amplified products were subsequently loaded onto an ABI PRISM 310 Capillary electrophoresis system (Applied Biosystems)for analysis. Electropherograms were analysed using the GeneMapper software. Each sample was amplified at least twice in both centres of analysis. The STR profile obtained in IT Sligo was subsequently compared against the STR profile generated in our second centre of analysis and against a staff elimination database.

2.5 Second centre verification

All molecular data from the adult individual included in this study was verified and replicated in an independent second centre facility in the University of Wisconsin (UW) using a second tooth from each skeleton sampled. Second centre analysis for the juveniles sampled was conducted in IT Sligo in a second aDNA laboratory located in a separate

building to the first centre analysis. This analysis was conducted by a second researcher up to six months after the first samples had been processed.

2.6 Criteria for Interpreting STR genotypes and authenticating sex of Ballyhanna adults

In order to avoid mistyping due to the presence of non-allelic peaks or sporadic contamination, an interpretation strategy was developed for authenticating the STR profiles and consequently the sex of the Ballyhanna adults. When comparing the molecular results from the adult teeth sampled in the two centres of analysis, the results were interpreted based on the following criteria.

2.6.1 Confirmed sex: To fully confirm the sex of an individual and conclude that the molecular sex was in accordance with the morphological sex, the results from the Quantifiler assay and the Powerplex assay (Amelogenin and four STR loci) needed to be in full agreement between both centres of analysis. In order to avoid mistyping STR profiles due to the presence of false alleles or sporadic contamination, an allele was only recorded in the consensus profile if it was observed at least twice in separate amplifications. The sex of such individuals was then compared against the morphological sex and these were assigned as true Males or Females. Artefact peaks present in the STR profiles which could be explained (i.e. stutter products) did not impact on the sex assessment.

2.6.2 Probable sex: In situations when only one molecular sexing method was in accordance between the two centres of analysis, the sex of such individuals was indicated as probable male or female. A probable sex was also assigned to partial STR profiles that could not be fully verified as a match to the second centre profile.

2.6.3 Inconclusive: When the sex and/or STR profiles identified using the two molecular methods were in disagreement between the two centres of analysis or did not correspond with the morphological sex, the sex of such individuals were assigned as "inconclusive". Also, if one STR marker in a sample was reported but did not match the corresponding sample from the second centre, such samples were also inconclusive.

Results

3.1 Validation of sexing assays using adult skeletal remains

The accuracy of our molecular sexing methods based on the SRY (Quantifiler Duo assay) and amelogenin gene (PowerPlex® S5 System) was tested and validated on 38 adult skeletons of known sex, 26 sexed as female and 12 sexed as male using osteological sexing methods (Bass., 1995). Where possible, both quantitative and STR assays were reproduced up to four times to confirm sexing and STR results from each skeleton. Of these 38 adult individuals, the morphological and molecular sex of 20 adults was in full accordance in the two centres of analysis. The STR profiles generated in each centre were also fully comparable. The molecular sex of 10 adults could not be confirmed due to poor amplification of the ancient DNA by either one or both assays and due to the strict interpretation criteria could therefore only be assigned a probable sex. The sex of 8 individuals was identified as inconclusive as the two sexing assays failed to detect sufficient amplifiable DNA.

The reproducibility of the results from the 20 individuals whose STR profiles and molecular and morphological sex were in full accordance determined that the methods used were valid. Thus these methods under the same criteria could be used to sex juvenile remains from Ballyhanna. Overall, the extraction technique and the two sex determination assays chosen for this study demonstrated a 53% success rate. However this low rate was more of a reflection on the preservation of the DNA within the skeletons sampled and the strict criteria

used to interpret the molecular sex, rather than the sensitivity and reliability of the molecular techniques used as part this study.

3.2 Determining the sex of the Ballyhanna juvenile remains

From assessing the results of both sexing methods in the two centres of analysis (see Table 2) the sex of four individuals, SK164, SK232, SK305 and SK870 were replicated at least twice in both centres and were thus identified as Males. The sex of 11 samples; SK10, SK53, SK56, SK319, SK536, SK668, SK687, SK730, SK772, SK859 and SK865 were also in accordance between the two centres, however as the results were not replicated more than twice for both sexing methods in either one or both centres, the sex of such individuals was indicated as probable male or female. According to our interpretation criteria, 10 of these individuals were thus sexed as probable males, while SK772 was sexed as a probable female.

The sex of four individuals, SK600, SK627, SK691 and SK694 were determined as inconclusive due to poor replication of the results in either one or both centres.

The measures taken against modern contamination appear efficient as all non template controls and extraction blank controls were negative. To prove the results were authentic, the STR profiles of each individual sampled were assessed between both centres of analysis. The veracity of the results was thus established as unique STR profiles were obtained for each sample. Each profile was compared against the staff elimination database which confirmed that the profiles were not contaminated by any of the personnel involved in the excavation or subsequent analysis of the remains. An inverse relationship between the amplicon product/allelic size and its quantity was observed throughout the analysis showing appropriate molecular behaviour, which is in accordance with ancient DNA authentication criteria.

Conclusion

As juvenile skeletal remains exhibit little sexual dimorphism, attempts to develop osteological techniques to determine the sex of individuals have been difficult. These methods are usually population dependent and as a consequence a number of studies demonstrated reduced accuracy when these methods were applied to other populations (Vlak et al., 2008; Cardoso & Saunders, 2008). It was for this reason that an alternative method was required to sex juveniles from Ballyhanna. By testing and validating the accuracy and reproducibility of our molecular sexing methods using osteoarchaeologically sexed adult remains, it was established that these methods could be used reliably on sex determination of individuals. As the only means of confirming the molecular sex of juveniles in this study was through reproducing the results in a second centre of analysis, extreme care had to be taken when interpreting the results. The possibility of a male individual being falsely identified as a female through allelic dropout of the Y chromosome was therefore always considered when interpreting the sex. It was noted when preparing juvenile teeth for extraction they were not as well preserved compared to the adult teeth. As a consequence, it seems that the aDNA was also less well preserved for some of the juvenile samples and thus made interpreting STR profiles a more difficult task due to stochastic and random sampling effects.

From the 19 juveniles sampled in the two independent aDNA centres, the results of 4 individuals were replicated at least twice for both sexing assays and were fully comparable between the two centres of analysis. According to the interpretation criteria, SK164, SK232, SK305, and SK870 were thus identified and confirmed as males.

Confirming the sex of the remaining 15 samples was more difficult due to poor replication and amplification of the ancient DNA by either one or both sexing assays. Adhering to the interpretation guidelines, the sex of 10 juveniles; SK10, SK53, SK56, SK319, SK536, SK668, SK687, SK730, SK859 and SK865 were sexed as probable males. SK772 was sexed as a probable female as the Quantifiler Duo assay detected only human

DNA in both centres. However the lack of signal from the SRY gene does not necessarily prove that the sample originated from a female source. This is specifically true for ancient degraded samples. The SRY gene cannot differentiate the presence of female DNA from an amplification failure as the SRY fragment is less informative than the amelogenin gene (Cunha et al., 2000). This was the rationale for using two DNA based sexing methods in this study and has been observed in other aDNA studies also (Cappellini et al., 2004; Cunha et al., 2000). The Amelogenin results for SK772 detected the X allele only but this was not replicated more than once in both centres of analysis. As duplication of every allele is required before it can be reported, confirming the sex of this individual was problematic as the presence of just the X allele could be a result of allelic dropout of the Y allele. Assigning a probable sex to this sample was the most appropriate solution. The sex of four individuals, SK600, SK627, SK691 and SK694 could not be confirmed due to poor replication of the results in either one or both centres of analysis and thus were determined as inconclusive.

The skeletal collection at Ballyhanna was representative of a typical population, with a 0.97:1 ratio of male to female adults which is close to the modern day sex ratio of 1.05:1 (Chamberlain, 2006). However, the mortality pattern at Ballyhanna suggested that fewer males were surviving to old age compared to females in medieval Ballyhanna and also revealed that more young males (17%) between 18-35 years died in Ballyhanna than females (15.3%) (McKenzie, 2012). The results from this study indicate a proportionally higher number of males among the juvenile remains analysed. Four of those analysed were sexed as males, 10 were probable males and one was a probable female. However, the number of juveniles sampled was small and thus these findings should be interpreted with caution.

In conclusion, the results from this study, although only representative of a small proportion of the entire juvenile population excavated from this cemetery, indicate an excess of juvenile male mortality in juveniles which could be due to a highermale mortality rate. For any firm conclusions to be drawn on the outcome of this analysis the sample number would have to be considerably expanded and further genetic sexing analysis conducted.

Acknowledgements

We thank theNational Museum of Ireland for giving us permission to sample the Ballyhanna skeletal remains. We also thank the National Roads Authority for providing the funding for this research and all members the Ballyhanna Research Project for their help and support throughout theproject.We are also grateful to Liza Kavanagh for conducting the second centre analysis on the juveniles.

Abbreviations

Ancient DNA (aDNA), amelogenin (AMEL), short tandem repeat (STR), real time PCR (RTPCR), University of Wisconsin (UW), Tuberculosis (TB).

References:

Akane, A., Seki, S., Shiono, H., Nakamura, H., Hasegawa, M., Kagawa, M., Matsubara, K., Nakahori, Y., Nagafuchi, S., and Nakagome, Y. (1992) 'Sex determination of forensicsamples by dual PCR amplification of an X-Y homologous gene' *Forensic Science International*, Vol. 52 (2):143-148.

Amory, S., Keyser, C., Crubezy, E., and Ludes, E. (2007) 'STR typing of ancient DNA extracted from hair shafts of Siberian mummies' *Forensic Science International* Vol. 166, 218–229.

Brickley, M. (2004) 'Determination of sex from archaeological skeletal material and assessment of parturition' *in* M Brickley & J I McKinley (eds), *Guidelines to the Standards for Recording Human Remains,* IFA Paper No. 7, 23-25. BABAO and IFS

Bramanti, B., Hummel, S., Chiarelli, B., and Herrmann B. (2003) 'Ancient DNA Analysis of the Delta F508 Mutation', *Human Biology,* Vol. 75, (1): 105-115.

Brothwood, M., Wolke, D., Gamsu, H., Benson, J., and Cooper, D. (1986) 'Prognosis of the very low birthweight baby in relation to gender', *Archives of Disease in Childhood,* Vol. 61, 559-564.

Butler, J. M. (2005) Forensic DNA Typing: Biology, Technology and genetics of STR markers, 2nd edition, Elsevier Academic press, New York.

Cappellini, E., Chiarelli, B., Sineo, L., Casoli, A., Di Gioia, A., Vernesi, C., Biella, M. C., and Caramelli, D. (2004) 'Biomolecular study of the human remains from tomb 5859 in the Etruscan necropolis of Monterozzi Tarquinia (Viterbo Italy)' *Journal of Archaeological Sciences,* Vol. 31 (5): 603-612.

Cardoso, H. F. V. (2008) 'Sample-specific (universal) metric approaches for determining the sex of immature human skeletal remains using permanent tooth dimensions', *Journal of Archaeological Science,* Vol. 35, 158-168.

Cardoso, H. F. V. and Saunders, S. R. (2008) 'Two arch criteria of the ilium for sex determination of immature skeletal remains: A test of their accuracy and an assessment of intra- and inter-observer error' *Journal of Archaeological Science,* Vol. 35 158-168.

Chamberlain, A.(2006), *Demography in Archaeology,* Cambridge University Press, Cambridge.

Cline, R. E.,Laurent, N. M,and Foran, D. R. (2003) 'The Fingernails of Mary Sullivan: Developing Reliable Methods for Selectively Isolating Endogenous and Exogenous DNA from Evidence', *Journal of Forensic Science,* Vol. 48, (2): 1-6.

Cooper, A., and Poinar, H. N.(2000) 'Ancient DNA: Do It Right or Not at All',*Science,* Vol. 289. (5482): 1139.

Cunha, E., Fily, M-L., Clisson, I., Santos, A. L., Silva, A. M., Umbelino, C., Cesar, P., and Corte-Real, A., Crubézy, E., and Ludes, B. (2000), 'Childrenat the Convent: Comparing Historical Data,Morphology and DNA Extracted from Ancient Tissues for SexDiagnosis at Santa Clara-a-Velha (Coimbra, Portugal)*' Journal of Archaeological Science,* Vol. 27,949-952.

Daskalaki, E., Anderung, C., Humphrey, L., and Götherström A. (2011) 'Further developments in molecular sex assignment: a blind test of 18th and 19th century human skeletons' *Journal of Archaeological Science,* Vol. 38, 1326-1330.

De La Cruz, I., Gonzalez-Oliver, A., Kemp, B. M., Roman, J. A., Smith, D. G., and Torre-Blanco, A. (2008)'Sex Identification of Children Sacrificed to the Ancient Aztec Rain Gods in Tlatelolco', *Current Anthropology,* Vol. 49, (3): 519-526.

DeWitte, S. N. (2010) 'Sex Differentials in Frailty in Medieval England' *American Journal of Physical Anthropology,* Vol. 143, 285-297.

Drevenstedt, G. L., Crimmins, E. L., Vasunilashorn, S., and Finch, C. E. (2008) 'The rise and fall of excess male infant mortality', *Proceedings of the National Academy of Sciences,* Vol. 105, (13): 5016–5021.

Faerman, M., Filon, D., Kahila, G., Greenblatt, C. L., Smith, P., Oppenheim, A., (1995) 'Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles' *Gene,* Vol. 167, 327-332.

Faerman, M.., Bar-Gal, K., Filon, D., Greenblatt, C. L., Oppenheim, S. A., and Smith, P. (1998) 'Determination of the sex of infanticide victims from the Late Roman Era through DNA analysis', *Journal of Archaeological Science,* Vol. 25, 861–865.

Falagas, M. E., Mourtzoukoua, E. G., and Vardakasa, K. Z. (2007) 'Sex differences in the incidence and severity of respiratory tract infections' *Respiratory Medicine,* Vol. 101, 1845-1863.
Gill, P., Whitaker, J., Flaxman, C., Brown, N., and Buckleton J. (2000) 'An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA', *Forensic Science International,* Vol. 112, 17- 40.
Gordon, E. C. (1991) 'Accidents among medieval children as seen from the miracles of six English saints and martyrs', *Medical History*, Vol. 35, 145-163.
Haas-Rochholz, H. and Weiler, G. (1997) 'Additional primer sets for an amelogenin gene PCR-based DNA-sex test', *International Journal of Legal Medicine*, Vol. 110, 312-315.
Hanawalt, B. A., (1977) 'Childrearing among the Lower Classes of Late Medieval England', *The Journal of Interdisciplinary History*, Vol. 8, (1): 1-22.
Hill, K. and Upchurch, D. M. (1995) 'Gender Differences in Child Health: Evidence from the Demographic and Health Surveys', *Population and Development Review* Vol. 21, (1): 127-151.
Holcomb, S. and Konigsberg, L. (1995) 'Statistical study of sexual dimorphism in the human fetal sciatic notch', *American Journal of Physical Anthropology,* Vol.97, 113-125.
Hummel, S., Bramanti, B., Schultes, T., Kahle, M., Haffner, S., and Herrmann, B. (2000) 'Megaplex DNA typing can provide a strong indication of the authenticity of ancient DNA amplifications by clearly recognizing any possible type of modern contamination', *Anthropologischer Anzeiger,* Vol. 58, (1): 15-21.
Kaestle, F. and Horsburgh, K. A. (2002) 'Ancient DNA in anthropology: methods, applications and ethics', *Yearbook of Physical Anthropology*, Vol. 45, 92–130.
Keyser-Tracqui, C., Crubézy, E. and Ludes, B. (2003) 'Nuclear and Mitochondrial DNA Analysis of a 2,000-Year-Old Necropolis in the Egyin Gol Valley of Mongolia',*American Journal of Human Genetics*, Vol. 73, 247–260.
Klein, S. L., (2000) 'The effects of hormones on sex differences in infection: from genes to behaviour', *Neuroscience and Biobehavioral Reviews,* Vol. 24, 627-638.
Lassen, C., Hummel, S., and Herrmann, B. (2000), 'Molecular sex identification of stillborn and neonate individuals ("Traufkinder") from the burial site Aegerten' *Anthropologischer Anzeiger*, Vol. 58, (1): 1-8.
Laupland, K. B., Church, D. L., Mucenski, M., Sutherland, L. R. and Davies, H. D. (2003) 'Population-Based Study of the Epidemiology of and the Risk Factors for Invasive *Staphylococcus aureus* Infections'*The Journal of Infectious Diseases*, Vol. 187, 1452–1459.
Lewis, M. E. (2002) 'The impact of industrialisation: comparative study of child health in four sites from medieval and post-medieval England (AD 850–1859)', *American Journal of Physical Anthropology,* Vol.119, 211–223.
Lewis, M. E. (2007) 'Difficult births, precarious lives' in *The Bioarchaeology of Children, perspectives from biological and forensic anthropology*, Cambridge University Press, 81-96.
MacDonagh, M., (2012) 'Introduction', *in*Murphy, E. M. and McKenzie, C. J. (eds.) '*The Science of a cemetery, The Ballyhanna Research Project'* Editorial solutions, Belfast.In press.
Macdonald, P. and Carver, N. (2012) 'Chronology, development and context—an analysis of the excavated graveyard at Ballyhanna', *in*Murphy, E. M. and McKenzie, C. J. (eds.) *'The Science of a cemetery, The Ballyhanna Research Project'*Editorial solutions, Belfast.In press.
Mannucci, A., Sullivan, K. M., Ivanov, P. L., and Gill, P. (1994) 'Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin' *International Journal of Legal Medicine,* Vol. 106, 190-193.

Matheson, C. D. and Loy, T. H.(2001) 'Genetic Sex Identification of 9400-year-old Human Skull Samples from Cayönu Tepesi, Turkey', *Journal of Archaeological Science,* Vol.28,569–575.
Mays, S. and Faerman, M. (2001) 'Sex identification in some putative infanticide victims from Roman Britain using ancient DNA', *Journal of Archaeology Science,* Vol. 28, 555–559.
McKenzie, C. J. (2012) 'The Adult Population at Ballyhanna'*in*Murphy, E. M. and McKenzie, C. J. (eds.) *'The Science of a cemetery, The Ballyhanna Research Project'*Editorial solutions, Belfast. In press.
Meyer, E., Wiese, M., Bruchhaus, H., Claussen, M. and Klein, A. (2000) 'Extraction and amplification of authentic DNA from ancient human remains', *Forensic Science International,* Vol.113, 87-90.
Migeon, B. R. (2007) 'Why females are mosaics, X-chromosome inactivation and sex differences in disease', *Gender Medicine,* Vol. 4, (2): 97-105.
Murphy, E. M. (2012) 'Lives Cut Short – Insights from the Osteological and Palaeopathological Analysis of the Ballyhanna Juveniles', *in*Murphy, E. M. and McKenzie, C. J. (eds.) *'The Science of a cemetery, The Ballyhanna Research Project'*Editorial solutions, Belfast. In press.
Naeye, R. L., Burt, L. S., Wright, D. L., Blanc, W. A., and Tatter D. (1971) 'Neonatal Mortality, The Male Disadvantage',*Pediatrics,* Vol. 48, (6): 902-906.
Nagaoka, T., Hirata, K., Yokota, E. and Matsuura, S. (2006) 'Paleodemography of a Medieval Population in Japan: Analysis of Human Skeletal Remains from the Yuigahama-minami Site', *American Journal of Physical Anthropology,* Vol.131, 1–14.
Nakahori, Y., Takenaka, O. and Nakagome, Y. (1991) 'A human X-Y homologous region encodes "amelogenin" ', *Genomics,* Vol. 9, (2): 264-269.
O' Rourke, D. H., Hayes, M. G. and Carlyle, S. W.(2000) 'Ancient DNA studies in physical anthropology', *Annual Reviews Anthropology,* Vol. 29, 217–242.
Pääbo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., and Hofreiter, M. (2004) 'Genetic Analyses from Ancient DNA', Annual Review of Genetics, Vol. 38, 645-679.
Resnick, D. and Niwayama, G. (1995) Osteomyelitis, septic arthritis, and soft tissue infection: organisms. *in* Resnick, D. (eds.), *Diagnosis of Bone and Joint Disorders,* third ed. W.B. Saunders, Edinburgh, 2448–2558.
Ricaut, F.-X., Fedoseeva, A., Keyser-Tracqui, C., Crubézy, E. and Ludes, B. (2005) 'Ancient DNA analysis of human Neolithic remains found in north-eastern Siberia', *American Journal of Physical Anthropology,* Vol. 126, 458-462.
Rivara, F. P., Bergman, A. B., Gerfo, J. P. and Weiss, N. S. (1982) 'Epidemiology of childhood injuries II: sex differences in injury rates', *American Journal of Diseases of Children,* Vol. 136, 502-506.
Rogaev, E. I., Grigorenko, A. P., Moliaka, Y. K., Faskhutdinova, G., Goltsovd, A., Lahti, A., Hildebrandt, C., Kittler, E. L. W. and Morozova, I. (2009) 'Genomic identification in the historical case of the Nicholas II royal family' *Proceeding of the National Academy of Sciences, USA,* Vol. 106, (13): 5258-5263.
Rohland, N., Siedel, H. and Hofreiter, M. (2010) 'A rapid column-based ancient DNA extraction method for increased sample throughput', *Molecular Ecology Resources,* Vol. 10, (4): 677-683.
Roth, E. A. (1992) 'Applications of Demographic models to paleodemography, *in* Saunders, S. R. and Katzenberg, M. A. (eds.),*Skeletal Biology of Past peoples: Research methods,* Wiley- Liss, Inc, 175-188.

Schofield, R. and Wrigley, E. (1979) 'Infant and child mortality in England in the late Tudor and early Stuart period', *in* Webster, C. (ed.) *Health, Medicine and Mortality in the Sixteenth Century*. Cambridge, UK: Cambridge University Press, 61-95.
Šlaus, M. (2000) 'Biocultural analysis of sex differences in mortality profiles and stress levels in the late medieval population from Nova Rača, Croatia', *American Journal of Physical Anthropology*, Vol. 111, 193–209.
Stone, A. C., Milner, G. R., Paabo, S., andStoneking, M. (1996) 'Sex Determination of Ancient Human Skeletons Using DNA', *American Journal of Physical Anthropology,* Vol. 99, 231-238.
Sullivan, K. M., Mannucci, A., Kimpton, C. P., and Gill, P. (1993) 'A Rapid and Quantitative DNA Sex Test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin', *Biotechniques,* Vol. 15, 636-641.
Vlak, D., Roksandic, M. and Schillaci, M. A. (2008) 'Greater Sciatic notch as a sex indicator in juveniles', *American Journal of Physical Anthropology,* Vol. 137, (3), 309-315.
Waldron, T., Taylor, G. M. and Rudling, D. (1999) 'Sexing of Romano-British baby burials from the Beddingham and Bignor Villas', *Sussex Archaeological Collections* Vol.137,71-79.
Walker, P. L., Bathurst, R. R., Richman, R., Gjerdrum, T. and Andrushkp, V. A. (2009) 'The causes of porotic hyperostosis and cribra orbitalia: a reappraisal of the iron-deficiency-anemia hypothesis', *American Journal of Physical Anthropology,* Vol. 139, 109-125.
Weaver, D. (1980) 'Sex differences in the *ilia* of a known sex and age sample of foetal and infant skeletons' *American Journal of Physical Anthropology,* Vol.52, 191-195.
Yang, D. Y. and Watt, K. (2005) 'Contamination controls when preparing archaeological remains for ancient DNA analysis', *Journal of Archaeological Science,* Vol. 32, (3): 331-336.