Incidence and characterization of polyglucosan bodies in the cerebella of Montserrat orioles (*Icterus oberi*)

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Abstract

Polyglucosan bodies are accumulations of insoluble glucose polymers and proteins that form intracytoplasmic inclusions in the brain, large numbers of which can be indicative of neurodegenerative diseases such as Lafora disease. Montserrat orioles (*Icterus oberi*) are an icterid passerine endemic to Montserrat with conservation populations maintained in captivity abroad. We demonstrate that polyglucosan bodies are unusually abundant in the cerebellar molecular and Purkinje cell layers and cerebellar peduncles of captive-bred and wild-caught Montserrat orioles. The bodies are periodic acid-Schiff positive and diastase resistant and label with concanavalin A and for ubiquitin, consistent with those seen in humans. We found no association of the polyglucosan bodies with any concurrent neurological lesions or clinical signs, nor are there *EPM2*A and *EPM2B* gene mutations associated with Lafora disease. We conclude that an abundance of cerebellar polyglucosan bodies may be a normal finding in aged Montserrat orioles and not a threat to the captive breeding population.

Keywords: Montserrat oriole, *Icterus oberi*, polyglucosan body, corpora amylacea, Lafora disease

Body

Polyglucosan bodies (PGBs) are non-membrane bound intracytoplasmic inclusion bodies comprising insoluble glucose polymers[15] found predominantly in the brain, where they may be neuronal, glial, or even extracellular, and in cardiac myofibers. PGBs are primarily composed of amylopectin-like, branched glucosans[5] with a much smaller fraction (4%) of assorted proteins, many of which were ubiquitinated, [4] and appear as round to oval, basophilic, periodic acid-Schiff (PAS) positive, diastase resistant, 2-20 µm globular inclusions. The prevalence of PGBs increases with age in humans and many other mammals in the absence of associated disease; these PGBs are usually termed “corpora amylacea” (“amyloid body”, a misnomer as they do not typically contain amyloid) and are considered a normal feature of ageing.[10] However, copious numbers of PGBs are key features of several severe, inherited human neurological diseases, including Lafora disease, caused by *EPM2A* or *EPM2B*

mutations,[8] and adult polyglucosan body disease, caused by *GBE1* mutations.[9] Lafora-like diseases have been reported in several animal species, including cows, cats, fennec foxes and parrots, but the genetic cause has only been determined in dogs, where there is a repeat expansion in *EPM2B.* [3,8,16] As these diseases show autosomal recessive inheritance, the presence of defective alleles is particularly detrimental in small populations where inbreeding is likely, such as endangered species in captive breeding programs.

Montserrat orioles (*Icterus oberi*), hereon referred to as orioles, are icterid passerines endemic to the Caribbean island of Montserrat.[1] Due to habitat loss associated with volcanic activity and invasive species, the species was listed as critically endangered.[1] Towards breeding and conservation goals, a captive population was established at Jersey Zoo (Durrell Wildlife Conservation Trust, DWCT) in the 1990s.[6,11] Wild populations have since stabilized, but the species is currently classified as Vulnerable by the International Union for Conservation of Nature (IUCN) Red List.[1] Estimates predict only 250-460 mature individuals remain in the wild,[1] so it is still crucial to maintain captive populations in good health.

In June of 2020, a post-mortem examination was performed on an aged oriole (case 1) from a population held at the Zoological Society of London's (ZSL) London Zoo, which had been euthanized due to age-related, degenerative osteoarthritis. While there was no clinical history of neurological disease, routine histological examination with hematoxylin and eosin unexpectedly revealed the presence of copious, 2-10 µm diameter, round to elongated, lightly basophilic, granular inclusions in the neuropil of the brain, but not in any other organ examined (spinal cord was not available). Further histochemical investigation revealed that the inclusions stained bright magenta with periodic acid-Schiff, which was not diminished by preceding diastase treatment. Inclusions were found randomly, in low numbers, throughout the brain but were most densely clustered in the molecular and Purkinje cell layers of the cerebellum (Figure 1a), where they formed linear arrays perpendicular to the layers (Figure 1b), and in the cerebellar peduncle (Figure 1c). Electron microscopy showed a

faintly targetoid, non-membrane bound body with a granular core and a homogenous outer layer (Figure 1d). There were no other findings in the brain.

The inclusions resembled PGBs but were present in very high numbers. There were 979 distinct PAS-stained inclusions in a single 0.1 mm^2 area of cerebellar molecular layer, as quantified by intensity thresholding and the "analyze particles" function of ImageJ in the green channel. This was likely an underestimate as many of the larger bodies were coalescing and so undercounted. While the presence of PGBs appeared incidental in this case, there was concern that this phenotype could be the consequence of heterozygous carriage of a mutant allele, as is seen in glycogen storage disease type 2 in humans, where carriers of the mutated GAA allele experience a mild, late onset version of the disease.[18] Such a lesion had not been previously reported in orioles or any other passerine; as the captive oriole population all descend from a founder population of just eight wild-caught individuals[11], we sought to investigate the incidence of PGBs in the captive population, to determine if they were associated with any clinical signs, neurological lesions or reproductive failure and to further characterize their composition and characteristics.

To survey the prevalence of PGBs in the captive population, brain tissue was collected from the archives of ZSL, DWCT and the International Zoo Veterinary Group (IZVG). Nineteen oriole brains with cerebellum were available for examination. Age at death ranged from 14 days to 20 years and 11 months old, with a median age of 3 years; there were eight males, six females and five animals of undetermined sex. Brains were scored by the presence of segmental clusters containing low (5-50), medium (50-200), or high (>200) numbers of PGBs, or as negative if only present in clusters of <5. PGBs were seen in eight animals(Table 1); of the six animals over eight years old, all were positive, and the youngest positive animal was 2 years and 8 months old, with low numbers of PGBs. Five of the positive cases were male and three were female. None of the cases had any history of neurological disease, all had definitively identified causes of death and no additional neurological lesions were identified histologically.

To further characterize the bodies, lectin- and immuno-histochemistry were performed to gain information on the carbohydrate sequences and assess the expression of neurodegenerative-associated proteins, respectively. The bodies were strongly labelled by concanavalin A (ConA) but not the β-galactose-specific peanut agglutinin (PNA), the N-Acetyl-D-glucosamine and sialic acid-specific wheatgerm agglutinin (WGA) or the *Aleuria aurantia* lectin (AAL). ConA, a mannose-specific lectin, is known to be able to bind to glycogen with a strong affinity. The ConA labelling was competitively inhibited by the presence of 0.5 M of methyl α-D-mannose (Figure 2a) confirming the specificity of binding via its carbohydrate binding site.

Using records from the Zoological Information Management System (Species360, zims.Species360.org), the relationships of the cases were deduced (Table 1). Case 4 was a wild-caught animal and cases 1, 2 and 7 were first generation offspring of two pairings of four other founders that were not available for this study. This suggests that if there is a genetic basis to the PGBs, it must be present in at least three of the eight founder individuals. As these animals were wild caught, their consanguinity is unknown, but these results suggest that predisposition to PGBs is common in the wild population. Lafora disease was considered unlikely in the cases examined due to the lack of clinical disease, the absence of typical radial filament appearance of the PGBs and the lack of PGBs in other organs. However, the concern remained that these animals may be heterozygotes for the condition. Human and canine Lafora disease is caused by mutations in either *EPM2A* or *EPM2B*, which respectively code for laforin, a carbohydrate phosphatase, and malin, a ubiquitin ligase, and are hypothesised to result in the precipitation of insoluble glycogen derivates.[7] To rule out the presence of such mutations, *EPM2A* and *EPM2B* were amplified from frozen skeletal muscle from case 1, and frozen muscle or liver from four additional unaffected controls, and sequenced. The coding sequence of *EPM2A* or *EPM2B* showed no insertions, deletions or premature stop codons, and there were no missense mutations in case 1 relative to the unaffected

controls. The coding sequences of the two candidate genes were therefore devoid of any putatively pathological mutations that could contribute to a Lafora-like phenotype.

Age is the most important correlate for high levels of PGBs in orioles, with them present in all animals over eight years old, and at high levels in all animals over 19. The maximum lifespan of wild Montserrat orioles is unknown but in other *Icterus* species it ranges from 6 to 14 years.[17] Thus, animals over 19 are likely senescent. It has recently been hypothesized that PGBs are transient organelles formed of a glucosan skeleton filled with ubiquitin-labelled cell waste, termed "wasteosomes".[14] Wasteosomes are normally excreted from cells and phagocytosed by macrophages for removal from the tissue.[13] Thus, the unusually high levels of PGBs in the brains of older orioles may represent reduced ability to excrete wasteosomes causing them to accumulate in axons, albeit harmlessly. However, wasteosomes can also contain exogenous waste, including DNA and protein of microorganisms.[12] Two of the eight positive animals in the study died from yersiniosis but it is not possible to say whether the infections were in any way affected by decreased wasteosome clearance, vice versa, or whether the findings are completely independent.

In summary, PGBs are unusually abundant in the cerebellum of aged orioles, both in wild-caught and captive-bred animals. The composition and histological appearance of the bodies resembles that of those seen in aged humans,[2] there are no associated neurological lesions or clinical disease and, in case 1, there is no evidence of association with *EPM2A* or *EPM2B* mutations. We conclude that PGBs are a normal, age-related finding in Montserrat orioles and do not pose a threat to the captive breeding of this vulnerable species.

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Declaration of conflicting interests

The authors declare no conflicting interests.

Authors' contributions

SS, MP and SF conceived and designed the study; SS, EW, MFS, DD and AB performed gross post mortem examinations; SS, MP, MFS, DD and ZJ performed histological examinations; DJE and AN performed ultrastructural examinations; KAB and YL performed lectin histochemistry; ZJ and AT performed immunohistochemistry; EW and EN provided technical support; ALS and SF performed genetic investigations; the manuscript was written by SS and MP with contributions from all authors.

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