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Unhatched eggs: methods for discriminating between infertility and early embryo mortality

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Unhatched eggs are a common phenomenon in birds and are often referred to as being 'infertile', which (confusingly) can mean at least two things: (1) that the ovum has not been fertilized or (2) that the embryo has died during development. These two broad categories of hatching failure can be difficult to distinguish, particularly in the early stages of embryo development. We describe methods to distinguish between infertility (due to insufficient sperm) and early embryo mortality in passerine eggs using the Zebra Finch *Taeniopygia guttata* as a model. We also describe how we successfully adapted these methods for use on eggs from a wild species, the Tree Sparrow *Passer montanus*, collected after the incubation period, and show that sperm can be visualized on the perivitelline layer of unhatched eggs even several weeks after laying.

Keywords: embryo mortality, infertility, parthenogenesis, Tree Sparrow, Zebra Finch.

Hatching success is an important component of avian reproduction and it is not unusual for one or more unhatched eggs to be found in nests after chicks have fledged (e.g. Koenig 1982). Confusingly, ornithologists often refer to unhatched eggs as 'infertile' when they show no sign of development (e.g. blood vessels or embryonic tissue) on visual inspection, and in general researchers rarely attempt to distinguish between eggs in which fertilization has occurred and those in which it has not. Further, since the contents of unhatched eggs incubated to full term are often 'addled' (i.e. yolk and albumen have amalgamated, and tissues have begun to decompose), field ornithologists generally assume such material cannot be usefully examined. Consistent with this view, Small *et al.* (2000) found that it was impossible to visualize sperm on the perivitelline layer (PVL) of Domestic Turkey *Meleagris gallopavo* eggs incubated for just 7 days, probably because the sperm cells had degraded (but see Wishart 1997).

Hatching failure results from two main causes; ova may fail to be fertilized, or fertilized eggs may fail to hatch. Fertilization is defined by the events that occur between penetration of the ovum by sperm and fusion of the male and female pronuclei (also referred to as syngamy). Embryonic mortality can occur at any stage of development and for a variety of reasons (Table 1). In eggs incubated for more than about 48 h, the presence of embryonic tissue and early blood vessel formation is usually sufficient to confirm that fertilization has taken place (Sellier *et al.* 2005). It is much more difficult to distinguish infertility (see Table 1, categories a–g) from early embryo mortality (EEM) that has occurred before any visible signs of embryo development are apparent (see Table 1, categories h and i). Studies of domesticated birds indicate that most embryo mortality is likely to occur either very early (including the period between fertilization and oviposition) or late in the incubation period, immediately prior to hatching (Lodge *et al.* 1971, Christensen 2001, Brun *et al.* 2004, Sellier *et al.* 2005).

Distinguishing between infertility and early embryo mortality is important for ornithologists interested in reproductive success (Bensch *et al.* 1994, Kempenaers *et al.* 1999, Morrow *et al.* 2002, Spottiswoode & Møller 2004), and has important implications for conservation (Gee 1983, Pletschet & Kelly 1990, Jamieson & Ryan 2000, Briskie & Mackintosh 2004). Infertility may be due to insufficient sperm (including, for example, sperm ejection by the female; Pizzari & Birkhead 2000), some defect of the sperm (which may in turn be a function of male health, condition,

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Table 1. Hatching failure categories and descriptions.

Status	Hatching failure categories
Infertility	(a) failure of the male to transfer sperm to the female
	(b) failure of the sperm to be stored in the female's sperm storage tubules
	(c) failure of stored sperm to be transported to the infundibulum (the site of fertilization)
	(d) failure of sperm in the infundibulum to interact with the perivitelline layer (PVL) of the ovum
	(e) failure of sperm to undergo the acrosome reaction and penetrate the PVL
	(f) failure of the sperm pronucleus to locate the female pronucleus
	(g) failure of the male and female pronuclei to fuse (syngamy)
Embryo mortality	(h) embryo mortality occurring between fertilization and egg-laying
	(i) embryo mortality occurring between egg-laying and the point where signs of embryonic development become apparent to the naked eye
	(j) embryo mortality between egg-laying and hatching
	(k) mortality during hatching

age, or contamination with toxic substances; Brillard 1990, Bakst & Wishart 1994, Bakst & Cecil 1997, Ackerman *et al.* 1999), or incompatibilities between the sperm and ovum (Zeh & Zeh 1997), resulting in biochemical rejection of sperm at the PVL. Technically, only a single sperm is necessary for fertilization in most organisms, but in birds, relatively large numbers are typically associated with the ovum at the time of fertilization, possibly because the female pronucleus within the germinal disc (GD) is a relatively small 'target' on the large, yolky ovum (see Birkhead *et al.* 1994, Steele *et al.* 1994). In the Domestic Fowl *Gallus domesticus*, Wishart (1987) showed that following a single insemination of 100 $\times 10^6$ sperm, the first ovum (surface area: 2828 mm^2) may have as many as 99 000 sperm associated with its PVL; successive ova had fewer sperm on their PVL, and by the time the number of sperm had fallen to about 200, an ovum had only a 50% chance of being fertilized. In the Zebra Finch *Taeniopygia guttata* – whose ovum is much smaller (surface area: 226 mm^2) than that of the fowl – the ovum had a 50% chance of being fertilized when 20 sperm were present on the PVL (Birkhead & Fletcher 1998).

Hatching failure due to embryo mortality may result from a variety factors, including exposure of eggs to variable temperature, humidity and microbial infection prior to the onset of full incubation (Meijerhof 1992, Cook *et al.* 2003, Beissinger *et al.* 2005, Cook *et al.* 2005), poor female condition or genetic incompatibility between the male and female, such as that resulting from inbreeding or outbreeding, including interspecific crosses (e.g. Lodge *et al.* 1971, Bensch *et al.* 1994, Etches 1996, Kempenaers *et al.* 1996, Kempenaers *et al.* 1996, Brillard *et al.* 1998, Christensen 2001, Sellier *et al.* 2005, Birkhead & Brillard 2007).

To date, few studies of wild birds have attempted to establish the cause of hatching failure (but see Birkhead *et al.* 1995, Svensson *et al.* 2007), whereas several studies of domestic birds have examined this issue in detail (e.g. Lodge *et al.* 1971, Kirby & Froman 1990, 1991, Bakst *et al.* 1997, 1998, Christensen 2001, Sellier *et al.* 2005). In the past, poultry biologists have claimed to be able to distinguish between infertile eggs and EEM either by candling eggs (see Sellier *et al.* 2005) or by visual inspection of the germinal disc in newly laid eggs, using criteria first described by Kosin (1944). However, more recent studies have questioned the accuracy of conclusions drawn by these methods, which may overlook cases of EEM (Bakst & Akuffo 2002, Sellier *et al.* 2006).

If eggs that are infertile or have experienced EEM are incubated to full-term, their contents often begin to decompose (as is the case with many unhatched eggs found in the wild), rendering methods such as those used by poultry biologists inapplicable, because they depend upon examining fresh eggs. Species in which hatching failure is high are sometimes endangered, so it may not be appropriate to sacrifice potentially viable eggs to get fresh material on which the available techniques may be applied. For this reason, it has been suggested that the techniques developed by poultry researchers have limited value in conservation research (Small *et al.* 2000).

The aim of the present study is two-fold. First, we present criteria for distinguishing unequivocally between the different causes of hatching failure (infertility and early embryo mortality) in passerine eggs. We describe methods for establishing these causes of hatching failure using the Zebra Finch as a

model passerine. Secondly, we test the applicability of these techniques on fully incubated, unhatched eggs from a wild species, the Tree Sparrow *Passer montanus*, to establish the amount of information that may be obtained from unhatched eggs remaining in nests after chicks have hatched.

METHODS AND RESULTS

Part 1: Criteria and methods for discriminating between the possible causes of hatching failure

The initial part of this study was conducted on captive, domesticated Zebra Finches. The birds were part of a long-established population maintained at the University of Sheffield since 1985, in which there is no evidence that the level of inbreeding is any different from that of a wild population (see Birkhead *et al.* 2006). The eggs of paired and unpaired females were used to obtain fertilized and unfertilized eggs, respectively. Unpaired females had been isolated from males for at least 6 months. Since the maximum duration of fertility in the Zebra Finch is around 13 days (Birkhead *et al.* 1989), these females could not have laid fertile eggs.

Eggs were taken within 1–2 h of oviposition and placed in an artificial incubator at 37.5 °C and examined after variable periods of time (0–120 h incubation) to establish the optimum duration of incubation before the eggs are examined using the methods described by Kosin (1944), Bakst and Akuffo (2002) and Sellier *et al.* (2006) (see Procedures, below). Our studies showed that incubation for 50–55 h provided an optimum compromise between: (1) sufficient development to be sure an egg was fertile and (2) sufficient disintegration of the PVL in fertile eggs to prevent the assessment of holes in the PVL (see below; also Jamieson & Ryan 2000). However, in eggs that were infertile or had undergone EEM at or before 50–55 h, the PVL maintained its integrity and could be examined after at least 120 h incubation and probably longer (see Methods, Part 2). This indicates that disintegration of the PVL may be linked to development rather than temperature.

To establish unequivocally the cause of hatching failure, three aspects of an egg must be examined: (1) the gross appearance of GD, (2) the presence or absence of sperm and holes on the PVL over the GD, and (3) the presence or absence of cell nuclei in the GD tissue.

The criteria (summarized in Table 2) for distinguishing between eggs of different status were:

Fertile and developing normally. The GD at 50–55 h of incubation typically comprises an embryo at the primitive streak stage (stage IV–V according to Hamburger & Hamilton's (1951) scheme; see Fig. 1A). At 50–55 h of incubation, blood vessels are not apparent (these first appear at 72 h and can be seen on candling). Holes and sperm are present on the PVL over the GD and large numbers of cell nuclei are present in the GD (Fig. 1D–F).

Table 2. Criteria for distinguishing between eggs of different fertility status. GD, germinal disc.

Status		GD appearance after 50–55 h incubation	Sperm/holes in PVL	Nuclei in GD
(a)	Fertile and developing normally	Embryo at stage IV–V (Hamburger & Hamilton 1951) – primitive streak formed See Fig. 1A	Present; including holes in PVL over GD	Abundant nuclei present Nuclei regular and round in appearance; sometimes elongated
(b)	Fertile but dead	Embryo at any stage up to IV–V (i.e. disparity between incubation period and developmental stage)	Present; including holes in PVL over GD	Nuclei present but relatively fewer than in a normally developing egg
(c)	Infertile	Dense white appearance No rings visible Vacuoles present; sometimes appears granular See Fig. 1B	None or very few (i.e. insufficient) sperm/holes in PVL	Nuclei absent (but see (d))
(d)	Parthenogenesis	Resembles an infertile GD See Fig. 1B	No sperm/holes in PVL	Small number of irregularly shaped nuclei present (Schut *et al.* 2008)

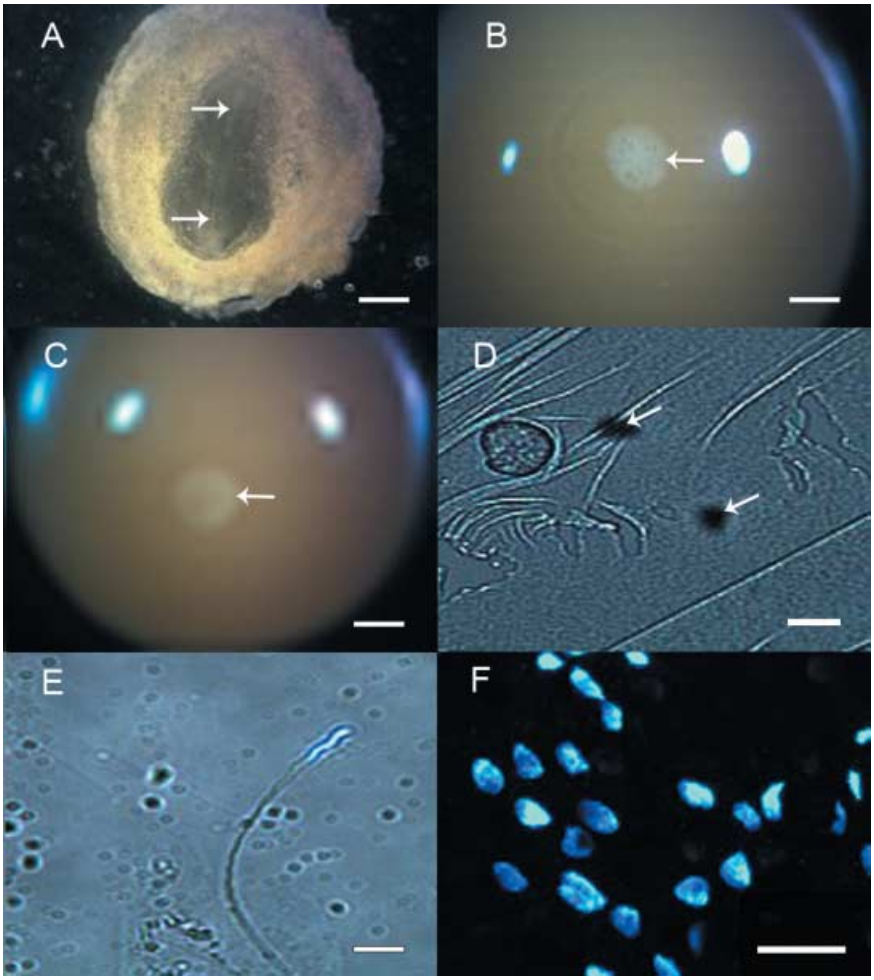

Figure 1. Zebra Finch. (A) Fertile germinal disc (GD) after 55 h of incubation showing primitive streak (arrows; scale bar = 1 mm). (B) Infertile GD after 55 h of incubation (scale bar = 1 mm). (C) Fertile, unincubated GD (scale bar = 1 mm). (D) Holes in the inner perivitelline layer (PVL) made by sperm entering the ovum (scale bar = 10 μm). (E) Sperm on outer PVL stained with Hoechst 33342 (scale bar = 10 μm). (F) nuclei from developing GD stained with Hoechst 33342 (scale bar = 10 μm). Note: Additional bright spots on yolk in (A–C) are reflections from the light-source.

Fertile but dead – early embryo mortality. The appearance of the GD after 50–55 h of incubation is quite variable, ranging from that of an unincubated egg (Fig. 1C) to that of an egg incubated for 50–55 h (see above). Sperm and holes are present in the PVL over the GD. Cell nuclei are present in the GD, their number depending on the stage of development at which embryo death occurred.

Infertile. The appearance of the GD at 50–55 h of incubation is distinctive, comprising a dense asymmetric whitish spot with no visible rings (Fig. 1B; cf. the unincubated, fertile egg in Fig. 1C). The GD of an infertile ovum often contains vacuoles, giving it a granulated appearance (Fig. 1B). However, the main criteria distinguishing an infertile ovum is the complete absence of sperm and holes in the PVL over the GD, and of nuclei from the GD. Occasionally parthenogenetic development occurs in infertile Zebra Finch eggs (Schut *et al.* 2008; see Table 2 (d)), and, when it does, the appearance of the GD at 50–55 h of incubation is similar to that of an infertile ovum. Parthenogenetic eggs have no holes in the PVL over the GD, but relatively small numbers of irregular nuclei are present in the GD (Kosin 1945, Schut *et al.* 2008).

Procedures

Here we describe methods for obtaining the required information on the GD and the PVL, together with a brief account of the relevant biology.

Germinal disc (GD)

Cut the egg open with fine scissors and discard the albumen. Place the yolk in a small Petri dish with the GD uppermost and examine using a low power (10–20X) binocular dissecting microscope illuminated with gooseneck lighting (e.g. Olympus KL 1500 halogen cold light source) fitted with blue filters to increase the contrast between the GD and surrounding yolk. Compare the GD with the images in Figure 1.

Fertilization occurs in the infundibulum (Olsen & Neher 1948). In chicken, turkey, and most passerines, including the Zebra Finch, the egg is laid (oviposited) 20–26 h after fertilization (Romanoff 1960, Eyal-Giladi & Kochav 1976, Gupta & Bakst 1993, T.R. Birkhead pers. obs.). Between fertilization and egg-laying the ovum is transported down the oviduct while the albumen and shell form around it (Romanoff 1960). During this same period the fertilized GD undergoes rapid cell division resulting in the formation of a blastoderm prior to egg-laying (Eyal-Giladi & Kochav 1976, Watt *et al.* 1993). In fertile eggs of the chicken, turkey, Zebra Finch, and probably most other avian species, the *area opaca* and the *area pellucida* are visible upon oviposition (Watt *et al.* 1993). The *area opaca* is a dense white ring in the periphery of the GD, whereas the *area pellucida* is the paler centre of the fertilized GD (Fig. 2).

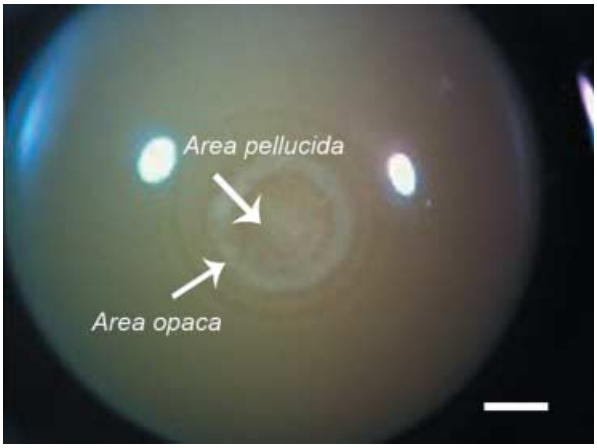

Figure 2. Fertile Zebra Finch egg after 24 h of incubation showing the opaque outer ring, the *area opaca*, and the pale inner area, the *area pellucida* (scale bar = 1 mm).

Nuclei in the GD

To check for the presence of cell nuclei, and hence the presence of a developing embryo, examine the GD as follows (from Gupta & Bakst 1993). Place a doughnut-shaped piece of filter paper, whose inner hole is about 1 mm larger in diameter than the germinal disc and whose outer ring is about 2 mm wide, on the yolk over the GD. Then, using a small pair of scissors, cut through the PVL around the outer edge of the filter paper ring. Using a pair of forceps, lift the filter paper with the PVL attached away from the yolk and place, with the GD uppermost, into a Petri dish containing phosphate-buffered saline (PBS). Using a hair-loop (i.e. a piece of human hair taped to a cocktail stick to form an oval loop about 2 mm across and 5 mm long), gently remove the excess yolk from the GD, which appears whitish when clean. Separate the GD from the PVL and place the GD on a microscope slide with 10 μL of Hoechst 33324 fluorescent dye (0.05 mg/ml). Disperse the GD material in the dye by gentle flushing in and out of a pipette, then add a coverslip and leave the slide to incubate in the dark for approximately 5 min. Examine the slide using a fluorescence microscope with a BP 340–380 excitation filter and a LP 425 suppression filter. Any nuclei present will be stained bright blue (Fig. 1F). Numbers of nuclei can be counted either systematically by scanning the slide (when numbers are low), or by counting fields of known area and extrapolating (when numbers are higher), to provide a crude index of the stage of embryo development (Kochav *et al.* 1980).

In normally developing eggs, cell division begins 6–8 h after fertilization (Eyal-Giladi & Kochav 1976, Watt *et al.* 1993) and oviposition occurs 16–18 h later. The fertile GD therefore contains numerous cell nuclei at oviposition and the number of nuclei continues to increase as development proceeds (Kochav *et al.* 1980, Liptói *et al.* 2004). In the Zebra Finch, the mean number of nuclei (± SD) per egg was highly variable and increased, as expected, with the duration of incubation (0 h eggs, 15565 ± 7755, $n = 9$ eggs; 24 h eggs, 26547 ± 7048, $n = 9$; 48 h eggs, 102812 ± 9444, $n = 7$). The shape and size of nuclei should be recorded to distinguish between normal and parthenogenetic development (Schut *et al.* 2008).

Holes and sperm on the PVL overlying the GD

Remove the PVL overlying the GD from the doughnut-shaped piece of filter paper and separate the inner and outer layers of the PVL by gentle agitation in PBS, teasing the layers apart with fine forceps. Examine the inner PVL using a light microscope with dark field optics to check for holes (left after sperm have entered the ovum; Bramwell *et al.* 1995; Fig. 1D). Place the outer PVL on a microscope slide with a drop of Hoechst dye and view in the same way as the GD material (see above) to visualize any sperm nuclei (Fig. 1E).

When sperm reach the ovum, the sperm plasmalemma binds to a sperm receptor on the outer surface of the inner PVL overlying the GD. The sperm then undergoes an acrosome reaction (Franklin 1970, Stepinska & Bakst 2006) and hydrolyses a path through the inner PVL leaving a hole. The sperm head enters the GD region of the ovum and is engaged by the microvilli formed by the oolemma. After a number of sperm have penetrated the ovum, the outer PVL is laid down, preventing the entrance of further sperm. Sperm that have not entered the ovum are trapped between the inner and outer PVLs (Wishart 1987).

Part 2: Applicability to unhatched eggs found in the field

The second part of this study was conducted on the Tree Sparrow, a small sexually monomorphic passerine in which high levels of hatching failure have been reported (Svensson *et al.* 2007). Our study population was one of those described by Svensson *et al.* (2007), located on organic farmland just outside Uppsala, Sweden. The present study was carried out in mid- to late June 2007 so all eggs were likely to have been from second clutches.

Unhatched eggs were removed from nests containing chicks aged greater than 2 days including nests from which chicks had fledged. Within Tree Sparrow broods hatching is synchronous, with most eggs hatching on the same day (Summers-Smith 1995), so all removed eggs were likely to remain unhatched. We obtained 40 unhatched eggs from 20 different pairs. By estimating the age of the live chicks in the nest (from overall body size, M. Svensson pers. comm.), and adding this to the incubation period (*c.* 12 days; Summers-Smith 1995), an estimate of the 'age' (i.e. period since being laid) of unhatched eggs was obtained. By the time of examination, the eggs were 15–25+ days old ($n = 40$) (after being laid) and had experienced either incubation or ambient temperatures, or both.

The unhatched Tree Sparrow eggs were kept in cool storage for 2–15 days to limit further deterioration. Originally, we intended to examine eggs using the techniques described above (Part 1). However, due to their age and possibly the effect of transportation to the lab, the egg contents had begun to degrade and the yolk and albumen had amalgamated (Fig. 3A), so these methods were not applicable. We therefore adapted our methods, as follows.

In all cases the yolk had disintegrated and become mixed with the albumen (Fig. 3A). This mixture was emptied into a Petri dish and a small amount of PBS added to dilute it. The remains of the degraded GD and PVL appeared as a small dense white bundle

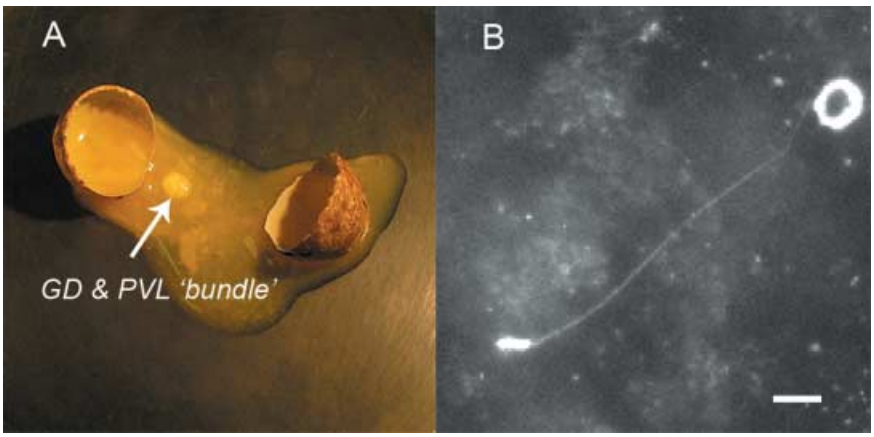

Figure 3. Tree Sparrow. (A) The contents of a fully incubated, addled tree sparrow egg on opening, showing the GD and PVL 'bundle'. (B) Sperm on PVL of fully incubated, addled egg (scale bar = 5 μm).

amongst the yolk and albumen mixture, visible with the naked eye (Fig. 3A). This bundle was removed using fine forceps and placed in PBS. The GD matter was then separated from the PVL under a dissecting microscope using fine forceps and a hair-loop. In most cases the GD was strongly adhered to the PVL and difficult to remove, meaning it was not always detached in its entirety. The removed GD matter was placed on a microscope slide with a drop of the fluorescent dye Hoechst 33342, and examined for the presence of any nuclei as described in Part 1.

The remaining PVL was then cleaned by washing gently in PBS and placed on a microscope slide. Probably as a result of degradation, the PVL left in the egg appeared to be only a small portion of the original entire PVL. As it was strongly attached to the GD, it is likely that this was the PVL overlying the GD. The PVL was examined in the same way as described in Part 1 to visualize and count any sperm present.

On opening, the egg contents were disintegrated in all but one egg, which contained an almost fully formed chick. No other eggs showed any obvious sign of development, although six eggs contained some dark red/brown matter that was strongly adhered to the GD, possibly including degraded blood vessels or embryonic tissue. Material from the first four eggs was not successfully salvaged and examined, due to initial difficulties in adapting the techniques. Sufficient GD and PVL material for examination was therefore obtained from 36 (90%) of the 40 eggs. In all of these eggs, nuclei and sperm were found in the GD material and PVL respectively. Sperm appeared similar to those found in the fresh eggs, with the head and midpiece still intact and tail attached in most cases (Fig. 3B), and the number of sperm was generally high (total number on the area of PVL above GD ranged from 21–202; mean = 88.87 ± 47.04 SD, n = 36). However, holes could not be visualized, presumably because of degradation of the inner PVL.

DISCUSSION

The methods we describe in Part 1 of this study can be used to distinguish between passerine eggs that are either infertile or have undergone EEM prior to, or during, the first 50–55 h of incubation. After 50–55 h of incubation the appearance of the GD alone can be used to identify eggs that have been fertilized and are developing normally. When a disparity exists between the duration of incubation and the appearance of the GD, EEM can be established from the presence of holes and sperm in the PVL, and the presence of nuclei in the GD. The fact that, in Tree Sparrows at least, sperm can be detected on the outer PVL, and nuclei detected in the GD, of fully incubated eggs (up to 25 days old followed by a further 15 days in cool storage) is an important finding, since it has previously been assumed that unhatched eggs incubated to full-term, whose contents have become 'addled', cannot provide useful information about fertilization (e.g. Small *et al.* 2000). The techniques described in Part 1 of this study, to examine fertility status of passerine eggs, were based on the yolk remaining intact and therefore not applicable to the Tree Sparrow eggs we examined in Part 2. However, the techniques in Part 1 were successfully adapted and used to yield similar results as they would on fresher eggs. Despite a certain degree of degradation of both GD and PVL matter, both nuclei and sperm could still be clearly seen under the microscope. The only apparent limitation to examining such addled eggs was that holes in the inner PVL could not be seen, presumably due to degradation of the inner PVL. Although it was not possible to examine the inner PVL for holes, it was still possible to infer whether fertilization had or had not occurred from the numbers of sperm present on the PVL. Birkhead and Fletcher (1998) found that the Zebra Finch ovum had a 50% chance of being fertilized when 20 sperm were present on the entire PVL; this number may be used as a 'threshold' value, i.e. with more than 20 sperm on the PVL and nuclei present in the GD, it is likely that fertilization has occurred. In contrast, with fewer than 20 sperm on the PVL, we cannot exclude the possibility that any nuclei found in the GD may be of parthenogenetic origin. This threshold number of sperm needs to be adjusted in different species depending on ovum size; birds with relatively larger or smaller ova (the yolk in a laid egg) will require relatively more or fewer sperm to achieve fertilization (Birkhead *et al.* 1994).

The efficacy of the techniques described in Part 1 decreases for fertile eggs as developmental stage advances; by the time blood vessels are beginning to form (around 72 h), the PVL has started to disintegrate, precluding its examination (although, by comparison, the developing embryo appears to retain much of its integrity). However, because disintegration of the PVL appears to be halted if development stops (that is, if EEM occurs), despite incubation continuing, a longer window of opportunity exists

for assessing the status of undeveloped eggs. Our data indicate that, for eggs that are not developing, it is still possible to establish the presence or absence of holes and sperm on the PVL after 4–5 days (96–120 h) of incubation and hence distinguish unequivocally between infertile and EEM eggs. Further, after a much longer period of incubation and/or ambient temperature (> 25 days), sperm on the PVL and nuclei in the GD are still visible; only the inner PVL has completely degraded by this time, so holes cannot be seen. These techniques would now benefit from further experimental validation; for example, by comparing fully incubated eggs (1) in which EEM has been experimentally induced with (2) those known to be infertile due to lack of sperm.

Our findings provide a protocol for researchers aiming to establish the causes of hatching failure in wild birds. If the species under investigation is of conservation concern, the best course of action may be to allow eggs to be incubated to full-term, removing unhatched eggs for examination after the other chicks in the brood have hatched, or a few days beyond the normal incubation period if no eggs hatch. Here we show that sufficient information can be obtained from such eggs to infer, with a reasonable degree of confidence, whether or not the eggs had been fertilized, from the presence of sufficient sperm on the PVL and nuclei in the GD. This method avoids the risk of sacrificing potentially viable eggs, but does not provide unequivocal evidence of sperm–ovum interaction (i.e. the presence of holes in the inner PVL).

If researchers wish to examine eggs for holes as well as sperm and GD nuclei, they may wait 4–5 days after incubation begins and then candle eggs for development. The rate at which embryos develop differs both between species (Sellier *et al.* 2006, T.R. Birkhead pers. obs.) and (obviously) with the intensity of incubation. However, examining eggs 4–5 days after laying should in most cases be sufficient to establish whether any development (i.e. through the presence of blood vessels) has occurred. This method provides a means by which researchers can minimize the risk of sacrificing a fertile, developing egg without waiting until the end of the incubation period. In other words, researchers should candle eggs 4–5 days after laying to determine (as far as possible) whether embryo development has occurred (Wilson 1994). If the egg shows no signs of development, the egg can be opened for more detailed examination. For eggs with unpigmented shells, candling is relatively easy, and it is straightforward to establish whether a developing embryo or associated blood vessels are present, indicating that fertilization has occurred. However, if eggshells are heavily pigmented, candling is more difficult, and the researcher faces a greater risk of sacrificing a viable egg.

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