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An Improved Method for Quantifying Hematozoa by Digital Microscopy

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ABSTRACT: Intensity of hematozoan infection is infrequently quantified because accurate calculations require visual counts of parasites relative to a large number of erythrocytes. Manual quantification of erythrocytes can be circumvented by using ImageJ software (developed by the National Institutes of Health) to count erythrocyte nuclei from digital images. Here we use the ratio of microscope erythrocyte counts to digital image erythrocyte counts (field:image ratio) to extrapolate erythrocyte counts from smaller digital images to the microscope's larger field of view. Field:image ratios were consistently calculated from 10 slides (resampling $P=0.049$) and used to rapidly estimate intensity of infection within 50,000 or more erythrocytes. Intensity of hematozoan infection calculated from manual quantification of 2,000 erythrocytes was significantly lower (0.46 times) than intensity calculated from digital quantification of 50,000 erythrocytes (bootstrap $P=0.02$). We contend that digital quantification of hematozoan infection offers a rapid and precise method to quantify infections of low to moderate intensity.

Key words: Birds, blood parasites, digital microscopy, hematozoa, ImageJ, infection intensity.

To determine epidemiology and pathogenicity of avian hematozoa, researchers frequently quantify the simple presence or absence of parasites (i.e., prevalence) within thin blood smears. However, intensity of hematozoan infection (number of parasites per standard number of erythrocytes) may be more ecologically relevant than prevalence of hematozoa (Fedynich et al., 1995). Recent research on birds has shown that hematozoan intensity is inversely related to energetic condition (Dawson and Bortolotti, 2000), return rates (Dawson and Bortolotti, 2000), and reproductive success (Allandar and Bennett, 1995; Merino et al., 2000; Marzal et al., 2005). Nonetheless, infection intensity is infrequently quantified because accurate calculations require labor-intensive, manual counts of erythrocytes that take approximately 30 min per 2,000 erythrocytes (Godfrey et al., 1987). Expedited methods of parasite quantification, such as counting parasites per unit scanning time (e.g., 10 min) or within a certain number of fields of view (e.g., 100), have been shown to be imprecise and irreproducible (Godfrey et al., 1987). As a result, intensity of infection can only be accurately quantified in relation to a specific number of erythrocytes scanned.

To alleviate tedious quantification of erythrocytes, Gering and Atkinson (2004) devised an automated method to count erythrocyte nuclei from digital images using ImageJ software developed by the National Institutes of Health (Rasband, 2006). Because digital images do not encompass the microscope's entire field of view, it is necessary to either 1) count hematozoa within the area captured in digital images or 2) count hematozoa within the entire field of view and correct for differences in area between digital images and fields of view. Since it is not possible to accurately delimit the area captured by digital images within a field of view, the former method entails manually counting hematozoa from real-time computer displays or digital pictures. Manual counts of parasites from digital images are more time consuming than direct counts of parasites using traditional microscopy because hematozoa are more difficult to identify within digital images. Quantification time is further increased by the

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number of images needed to compensate for a microscope's larger field of view (e.g., 50,000 erythrocytes are visible within 525 digital images or 175 fields of view).

We suggest that by counting erythrocytes within digital images and correcting for differences in area between digital images and fields of view, intensity of infection can be quickly calculated from a large number of erythrocytes (e.g., >20,000). Differences in area between digital images and fields of view cannot be directly quantified because slide micrometers are not precise enough to accurately estimate the area encompassed by a digital image or field of view. Instead, we extrapolated erythrocyte counts from digital images to fields of view by calculating the ratio of microscope erythrocyte counts to image erythrocyte counts (hereafter the field:image ratio). Here we demonstrate that digital quantification of infection intensity within 50,000 erythrocytes more precisely estimates infections of low to moderate intensity when compared to traditional quantification of intensity within 2,000 erythrocytes.

Yellow-rumped Warblers (*Dendroica coronata;* $n=209$), Magnolia Warblers (*Dendroica magnolia;* $n=204$), and Yellow Warblers (*Dendroica petechia;* $n=216$) were captured using 2.6×12 m mist nets in northwestern Ohio during the spring migratory period of 2004 and 2005 (16 April to 3 June). We used a sterile 27-gauge needle to puncture the brachial vein and collect 10–30 μL of blood in preheparnized microhematocrit capillary tubes. A drop of blood was immediately placed on two clean glass slides to prepare blood smears. At the end of each day, blood smears were fixed in 100% methanol for 1 min. Blood smears were stained for 10 min with Jenner's Stain pH ~7 and 60 min with Giemsa Stain pH ~7 (The Ohio State University Reagent Lab, Columbus, Ohio, USA).

An Olympus BX40F microscope mounted with a Spot RT Color digital camera (Diagnostics Instruments Inc., Sterling Heights, Michigan, USA) was used to examine blood smears at 1,000× under oil immersion. Because hematozoans may be heterogeneously distributed throughout blood smears (Godfrey et al., 1987), examination began at a randomly selected starting position where the smear formed a relatively homogeneous monolayer of cells. Blood smears were examined until at least 50,000 erythrocytes were scanned for parasites. Thirty-six slides ($n=7$ Magnolia Warblers; $n=27$ Yellow-rumped Warblers, $n=2$ Yellow Warblers) with at least one intracellular parasite ($n=29$ *Haemoproteus* spp., $n=2$ *Plasmodium* spp., $n=5$ unidentified *Haemoproteus* or *Plasmodium* spp.) were randomly selected for manual and digital quantification of infection intensity. Manual quantification of intensity was calculated from manual counts of parasites within 2,000 manually counted erythrocytes (four to 11 fields of view). Digital quantification of intensity was calculated from manual parasite counts and simultaneous image capture of 150 to 175 fields of view (at least 50,000 erythrocytes).

Digital photographs of each examined field of view were taken using Spot Advanced software (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA) with automatically adjusted color balance and exposure. Erythrocyte images were taken with gain=2 to prevent particulate and other nonnucleic erythrocyte matter from being counted as erythrocytes (compare Figs. 1a and 1b). Quantification of nucleated erythrocytes from digital images was performed using ImageJ software (Rasband, 2006). Image series were imported into image J (Fig. 1b), converted to 8-bit formats, and subjected to automatic thresholding (Fig. 1c) (Rasband, 2006). The "Analyze Particles" function was used to count erythrocyte nuclei as objects between 1,000 and 4,200 pixels in size (Fig. 1d) (Rasband, 2006). Nuclei located on the edge of digital images were excluded to avoid overdispersion (Godfrey et al., 1987). Intensity of infection is reported

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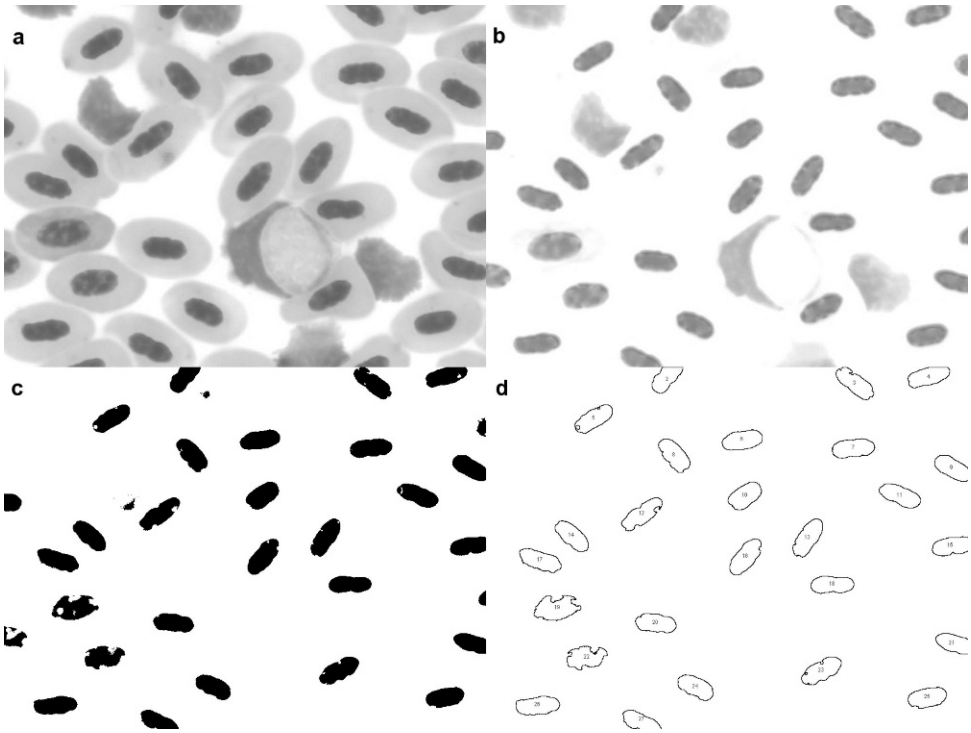


FIGURE 1. One quarter of a digital image from a single avian blood smear (1,000× under oil immersion) demonstrating erythrocyte quantification using ImageJ software where (a) image gain = 1, (b) image gain = 2, (c) image b after 8-bit conversion and automatic thresholding, (d) image c after "analyze particle" function identified erythrocyte nuclei 1,000 to 4,200 pixels in size.

as number of parasites per 2,000 erythrocytes (Godfrey et al., 1987).

Statistical analyses were performed using R Version 2.2.1 (R Development Core Team, 2005). Field:image ratios were calculated for each of 36 slides by dividing manual erythrocyte counts by erythrocyte counts using ImageJ. A bootstrap analysis of mean field:image ratio was performed to calculate 95% confidence intervals for theoretical samples of one to 25 slides. To determine the repeatability of field:image ratio calculation from 10 slides (2,000 erythrocytes per slide), field:image ($n=36$) ratios were resampled as 10,000 pairs of 10. The number of significant pairwise differences (analysis of variance $P<0.05$) was divided by the number of trials ($n=10{,}000$) to estimate the likelihood of obtaining significant differences between mean field:image ratios calculated from 10 slides. Intensity of infection from image analysis was calculated using the equation: (no. parasites×2,000)/(no. erythrocytes from images×mean field:image ratio). A bootstrap analysis of scaled differences of intensity (difference/mean) was used to determine if manually quantified intensity (manual counts of 2,000 erythrocytes; $n=32$) was different than digitally quantified intensity (digital counts of 50,000 erythrocytes).

The mean field:image ratio for 36 slides was 2.99 (SE=0.05). Bootstrap analysis of mean field:image ratios revealed that 95% confidence bounds approached asymptotic lines and ceased exponential reduction at approximately 10 slides (Fig. 2). Probability of pairwise differences in mean field:image ratios from 10 slides was 0.049, thus demonstrating that erythrocytes from digital images can be consistently extrapolated to larger microscopic fields of view.

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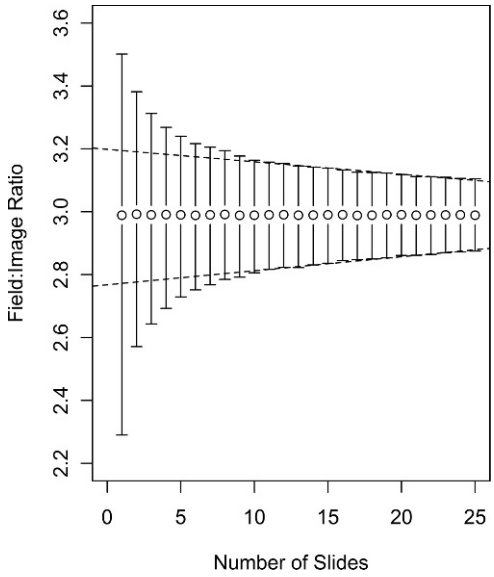


FIGURE 2. Mean field:image ratio (manual erythrocyte count/digital erythrocyte count) with 95% confidence intervals based on bootstrap calculations across theoretical samples of one to 25 slides. Dashed lines approximate horizontal asymptotes.

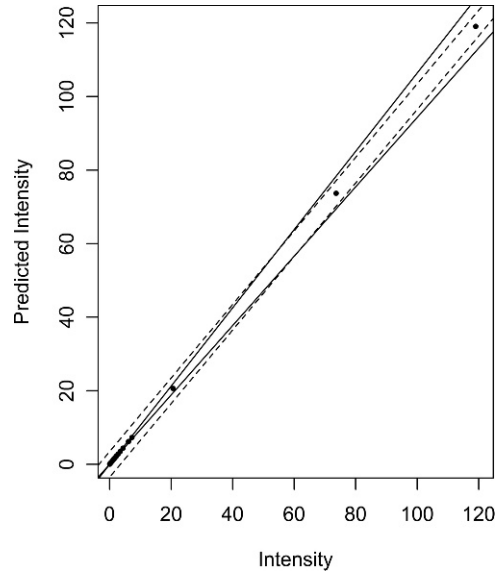


FIGURE 3. Estimated hematozoan intensity 95% confidence bounds using standard errors from mean field:image ratios for 10 slides (solid line, SE=0.18) and mean difference between manually and digitally quantified intensity (dashed line, SE=1.71). Confidence bounds intersect at intensity=58. Points represent intensity of hematozoan infection from examined blood smears (n=32).

For low to moderate intensity infections (<58 parasites/2,000 erythrocytes) the variability surrounding field:image ratios from 10 slides (SE=0.18) resulted in narrower 95% confidence bounds of predicted intensity than confidence bounds based on variability between manual and digital quantification of intensity (SE=1.71; Fig. 3). Mean scaled difference of intensity from bootstrap analysis was 0.46 less than zero (P=0.02, 95% CI −0.91 to −0.02); therefore infection intensity from manual quantification of 2,000 erythrocytes was significantly lower than intensity from digital quantification of 50,000 erythrocytes.

Quantifying intensity of infection using a large number of manually counted erythrocytes (e.g., 10,000 or 20,000) would offer the most accurate and precise means of quantifying infections of varying intensities, but the length time required to count large numbers of erythrocytes renders manual quantification impractical. Calculation of field:image ratios from manual counts of 20,000 erythrocytes (2,000 erythrocytes across 10 slides) divided by ImageJ counts of erythrocytes from digital images requires a one-time investment of approximately 6 hr. Subsequent calculation of infection intensity using ImageJ and field:image ratio will allow researchers to estimate intensity of infection from 50,000 erythrocytes within 30 to 40 min per slide instead of approximately 12 hr.

Our data reveal that hematozoan infections of low to moderate intensity are more precisely estimated using digital quantification of parasites within 50,000 erythrocytes than traditional manual quantification of parasites within 2,000 erythrocytes (Fig. 3). This difference in precision likely resulted from heterogeneity in dispersion of parasites and the lower likelihood of detecting parasites within 2,000 erythrocytes (12 of 32 slides). Likewise, nondetection of parasites biased our estimation of intensity from manual

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counts and resulted in lower measures of intensity from manual counts of 2,000 erythrocytes when compared to digital counts of 50,000 erythrocytes (bootstrap $P=0.02$). Because infections of high or even moderate intensity are uncommon (in this study three of 31 individuals had intensity >eight parasites per 2,000 erythrocytes), digital quantification of erythrocytes with simultaneous manual quantification of hematozoa in blood smears offers a repeatable means of calculating intensity for the majority of infections.

We have provided evidence that field:image ratios can be consistently calculated from simultaneous digital and manual quantification of 2,000 erythrocytes from 10 slides (Fig. 2). However, since no two setups for digital microscopy are exactly the same, we recommend that field:image ratios are calculated for each microscopy setup. While any common, quantifiable, and randomly distributed particle or cell would be expected to yield consistent field:image ratios, ratios should be calculated for each taxa of interest until data are published on the repeatability of field:image ratios in relation to cell size.

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