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Using quantum dots as pollen labels to track the fates of individual pollen grains

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Abstract

1. Despite a long history of significant advances in understanding natural selection and evolution, the field of plant reproductive biology has largely studied plant mating without directly tracking pollen movement due to a lack of suitable pollen-tracking methods.
2. Here, we develop and test a novel pollen-tracking technique using quantum dots as pollen-grain labels. Quantum dots are semiconductor nanocrystals that are so small, they behave like atoms. When exposed to UV light, they emit extremely bright light in both visible and infrared wavelengths. We tested the suitability of non-toxic $CuInSe_xS_{2-x}$/ZnS (core/shell) quantum dots with oleic-acid ligands as pollen-grain labels. Using a micropipette, we dispensed quantum dots dissolved in hexane in minute volumes (0.15–0.5 μl) directly onto dehisced anthers of four different plant species from four different families (*Wachendorfia paniculata* [Haemodoraceae], *Sparaxis villosa* [Iridaceae], *Arctotheca calendula* [Asteraceae], *Oxalis purpurea* [Oxalidaceae]).
3. After application, the hexane solvent evaporated immediately, leaving behind quantum dots that remained attached to pollen grains of the four different plant species even after agitation in a polar solvent. This suggests a lipophilic interaction between oleic-acid ligands on quantum dots, and pollenkitt surrounding pollen grains. We also showed that most pollen grains within anthers of the same four plant species were labelled with quantum dots after applying a volume of quantum-dot solution sufficient to cover an individual anther. To test whether quantum-dot pollen labels influenced pollen transport, we conducted pollen transfer trials (one donor, 10 sequential recipients) on *S. villosa* using captively reared honey bees to ensure bees were free of external pollen prior to experiments. We found no difference in pollen transport to recipients from donor flowers with labelled or unlabelled pollen grains.
4. We demonstrate that quantum dots can be used as pollen labels allowing subsequent tracking of pollen fates. This method is relatively inexpensive (<$500 for equipment and ca. $0.02 per labelled anther thereafter) and can be simply and directly applied to anthers of most flowers in the lab and field. The ability to track pollen grain movement in situ, may help to address a historically neglected aspect of plant reproductive ecology and evolution.

KEYWORDS
bio-labels, fluorescent markers, pollen dispersal, pollen fates, pollen tracking, pollen transfer, pollination, quantum dots

1 | INTRODUCTION

Flowers have evolved primarily to facilitate pollen movement from anthers to stigmas. Yet, despite over a century of work on pollination biology, our limited ability to track the movement of pollen grains has rendered pollen movement one of the most poorly studied aspects of plant reproductive biology (Minnaar, Anderson, de Jager, & Karron, 2019). While increasingly affordable and accurate paternity analyses allow us to identify which plants sired which seeds (Jones, Small, Paczolt, & Ratterman, 2010), this information may confound the outcomes of pollen dispersal with effects of fertilisation and seed-development processes. A complete understanding of floral function, evolution and ecology requires the ability to track all pollen grains, not just those that successfully fertilise ovules.

On average, fewer than 2% of pollen grains produced by flowers successfully reach conspecific stigmas (Gong & Huang, 2014; Harder & Thomson, 1989). The remaining 98% may be lost to multiple potential fates (Inouye, Gill, Dudash, & Fenster, 1994), with each avenue of pollen loss representing an opportunity for selection to act on traits that reduce the amount of wasted reproductive potential (Harder & Barrett, 1996; Harder & Thomson, 1989; Minnaar et al., 2019). Since most pollen is lost during transport of pollen from anthers to stigmas, it represents an important phase for selection on plant reproductive traits; however, this phase of reproduction remains poorly studied, with most studies unable to directly link post-fertilisation measures of mating success to selective agents that act on traits that facilitate pollen transport (Minnaar et al., 2019).

The lack of suitable techniques to track pollen has similarly limited our understanding of important ecological aspects of pollination. For example, the importance of specific pollinators in facilitating reproduction and persistence of plant species in co-flowering communities is most often assessed through pollinator visitation patterns. However, visit frequency is a poor proxy of pollen transfer efficacy (King, Ballantyne, & Willmer, 2013). Determination of the actual ecological importance of specific pollinators to different plant species should ideally incorporate direct assessments of pollen transfer between unmanipulated donors to several unmanipulated recipients. At present, this is only possible for a small subset of plant species. Reliable data on pollinator importance may be vital to conservation efforts in vulnerable ecosystems. In pollination-dependent agricultural systems, the ability to track pollen movement and determine pollination efficacy would allow more accurate valuation of pollination services provided by both managed and natural crop-flower visitors. Such direct assessments are currently lacking (Rader et al., 2009), but vital in informing and encouraging sustainable management of globally declining wild and managed populations of flower-visiting animals (Potts et al., 2016).

1.1 | Pollen-tracking methods to date

While generally applicable pollen-tracking methods remain limited, successful pollen tracking has been achieved for a small proportion of flowering plant species. Most of the success in tracking pollen comes from a single plant family, the Orchidaceae. Orchid pollen grains are contained in large pollen packets called pollinaria, which can be stained using histochemical dyes and observed directly in the field using a hand lens (Peakall, 1989) or labelled using uniquely coded microfilm tags (Nilsson, Rabakonandrianina, & Pettersson, 1992). The dyed massulae (subunits of a pollinarium) can then be recovered and counted from other flowers once transferred (Johnson & Harder, 2018; Peakall, 1989). However, most angiosperms (ca. 98% of all families) produce granular pollen (i.e. monads, dyads and tetrads) (Harder & Johnson, 2008), and attempts at staining entire anthers containing granular pollen with histochemical dyes (safranin and methyl green) have largely failed, as these dyes require the addition of water to entire anthers, leading to altered pollen presentation through hydrophobic pollen clumping, and poor dye absorption. Consequently, to our knowledge, this method of pollen staining has only been used three times (Armbruster, Shi, & Huang, 2014; Huang & Guo, 1999; Huang & Shi, 2013). Pollen grains have also been labelled with radioactive elements (Colwell, 1951), neutron-activated elements (Gaudreau & Hardin, 1974; Handel, 1976) and ^{14}C labels (Pleasants, Horner, & Ng, 1990; Reinke & Bloom, 1979). However, concerns about environmental exposure to radioactive labels, the complicated and time-consuming process of detection of neutron-activated and ^{14}C labels (up to 14 weeks) and the limited number of unique labels (usually just one), ultimately rendered these methods ineffective.

Instead of attempting to directly label pollen grains, some researchers have used fluorescent dye particles as a pollen proxy (Price & Waser, 1982; Stockhouse, 1976; Waser & Price, 1982). In some cases, fluorescent dye particle deposition on stigmas correlates relatively well with pollen grains deposited per visit (Fenster, Hassler, & Dudash, 1996; Van Rossum, Stiers, Van Geert, Triest, & Hardy, 2011; Waser & Price, 1982). However, pollen grains are often found on stigmas when dye is not, or dye is present when pollen is not (Waser & Price, 1982). In other studies, dye particles significantly over- or underestimated pollen transfer (Adler & Irwin, 2006; Campbell, 1991; Thomson, Price, Waser, & Stratton, 1986; Waser, 1988). Micronised metal (Zn and Sn) dusts have also been applied to dehisced anthers (Wolfe, Estes, & Chissoe, 1991). While some of the metal particles labelled grains directly, their presence on pollen grains was likely superficial. Moreover, to detect metal dust particles, samples have to be gold plated for subsequent scanning

electron microscopy. To our knowledge, this method has never been applied outside of the original study that reported it.

In rare cases, intraspecific colour variations of pollen grains have been used to track pollen (e.g. Holsinger & Thomson, 1994; Thomson & Plowright, 1980). Similarly, intraspecific variation in pollen size, associated with different anther levels in heterostylous morphs, has been exploited to quantify pollen movement (Harder & Barrett, 1995; Nichols, 1985; Stone, 1995). Unfortunately, these methods are limited in their applicability, as most other systems do not have pollen-colour or -size polymorphisms that can be exploited. More recently, researchers have been able to genotype individual pollen grains using microsatellite markers (Matsuki, Isagi, & Suyama, 2007) allowing researchers to identify the individual plant origin of pollen grains found on floral visitors (Hasegawa, Suyama, & Seiwa, 2009, 2015; Matsuki, Tateno, Shibata, & Isagi, 2008) and stigmas (Hasegawa et al., 2009). However, this technique is labour intensive and expensive, requiring careful pollen isolation, DNA extraction and sequencing of individual pollen grains. To make quantitative assessments of various aspects of the pollen export process more practical, we have developed an easy and relatively inexpensive method that can be applied in the field.

1.2 | Quantum dots as potential pollen labels

Quantum dots are extremely small nanoparticles of semiconductor metals. They range in diameter from 2 to 10 nm (10–50 atoms across) (Neeleshwar et al., 2005) causing atom-like confinement of electrons and electron-holes in bound discrete states (Gammon, 2000). When relatively large semiconductor metal objects are excited (e.g. when an electrical charge is applied), electrons bound to atoms (in the valence band) become free (jump to the conduction band) and can move within the crystal lattice of a large semiconductor object (i.e. electrical conduction) (Cho, 1979; Dean & Herbert, 1979). Electrons behave in this way as long as the semiconductor object is large relative to the wavelength of the electrons (Brus, 1984). In contrast, when the semiconductor object is reduced to the nanoscale (quantum dots), the valence and conduction energy bands that the electrons can occupy become discrete (Yoffe, 2001). In a spherical quantum dot, this can be theoretically visualised as a ball with discrete layers: the outer layer is the conductance band, and the inner layer is the valence band. When electrons become excited (usually through UV radiation), they jump from the valence band to the conduction band (Ekimov, 1991), leaving behind an electron-hole (a quasiparticle with a positive charge relative to electrons) (Yoffe, 2001). Normally, in a large semiconductor object, electrons can move freely, independent of the electron-hole. But, because of the tight confinement of electrons, the close proximity of the electron and the electron-hole results in the formation of an exciton—an electron–electron-hole pair in a bound state—which jumps to the conduction band (Cho, 1979; Dean & Herbert, 1979; Kusrayev, 2008). When the exciton returns to the ground state (valence band), it emits light energy, causing the quantum dot to fluoresce (Ekimov, 1991). Therefore, quantum dots can emit bright light in the visible spectrum when excited with UV radiation (Brus, 1984; Ekimov & Onushchenko, 1981, 1982). The size of the quantum dot determines the radius of the two energy bands, and therefore the exciton's light emission wavelength (Yoffe, 2001). The emission colour of quantum dots can therefore be tuned precisely by altering the size of the quantum dot.

Quantum dots were first employed as bio-labels two decades ago (Bruchez, Moronne, Gin, Weiss, & Alivisatos, 1998; Chan & Nie, 1998) and offer several advantages over traditional bio-labels (Jaiswal & Simon, 2004): (a) Their emission colour can easily be manipulated to specification by controlling the size of quantum dots produced. (b) They have much greater photostability than traditional fluorescent markers which lose their fluorescence comparatively quickly under excitation. (c) They have very large Stokes shifts (difference between excitation and emission wavelengths) and, therefore, multiple different coloured quantum dots can be excited by a single light source and detected simultaneously. (d) The ease with which bio-functional groups can be attached to quantum dots allows them to be used as potential bio-labels for virtually any biomolecule, including pollen grains.

Initially, quantum dots were made from toxic heavy-metal semiconductor cores (primarily Cadmium), which precluded their use in natural environments (Hardman, 2006). However, several commercially available, non-toxic alternatives, have recently been developed (Xu et al., 2016). Among these, CuIn-core quantum dots have achieved the highest quantum yields and therefore brightness, and many formulations are now commercially available. Most quantum dots are produced with oleic-acid ligands that allow them to be dissolved in non-polar solvents. Since oleic acid is lipophilic, it may bind to lipid-rich pollenkitt (Pacini & Hesse, 2005) surrounding pollen grains, allowing direct attachment of quantum dots to pollen grains.

Here, we evaluate the potential of quantum dots as pollen labels using three important functional criteria: (a) quantum dots must attach directly to pollen grains; (b) the application of quantum dots to an anther should result in most pollen grains being labelled; (c) quantum-dot labels should not affect pollen grain placement on pollinators and subsequent deposition on stigmas.

2 | MATERIALS AND METHODS

2.1 | Proposed method

2.1.1 | Quantum dots

For all experiments, we used heavy-metal-free $CuInSe_xS_{2-x}$/ZnS (core/shell) quantum dots (UbiQD, Los Alamos—currently distributed through Strem Chemicals, Newburyport) with zinc oleate ligands (zinc complex with oleic acid). At the time of writing, these quantum dots were commercially available in four colours in the visible range with peak fluorescence (±10 nm) at 550 nm (green), 590 nm (yellow), 620 nm (orange) and 650 nm (red). We dissolved quantum dots in hexane to make a dispensable quantum-dot solution. The concentration and volume of quantum-dot solutions applied to anthers were tailored to suit pollen and anthers of each

plant species tested (see below). Quantum-dot solutions were stored in complete darkness below 30°C inside small 2 ml clear glass vials (9 mm thread; 12 × 32 mm, product number—29371-U; Supelco, Bellefonte, PA) closed with plastic caps containing PTFE/silicone septa (9 mm polypropylene cap; PTFE/silicone septum; product number—29319-U; Supelco). The vial septum composition is necessary for safe long-term storage of quantum-dot solutions as non-PTFE/silicone septa and plastic caps are eroded by hexane fumes.

2.1.2 | Quantum-dot application to pollen

We applied quantum dots directly to individual dehisced anthers using a micropipette (0.1–2.0 µl; product code—p3942-2; Biopette,

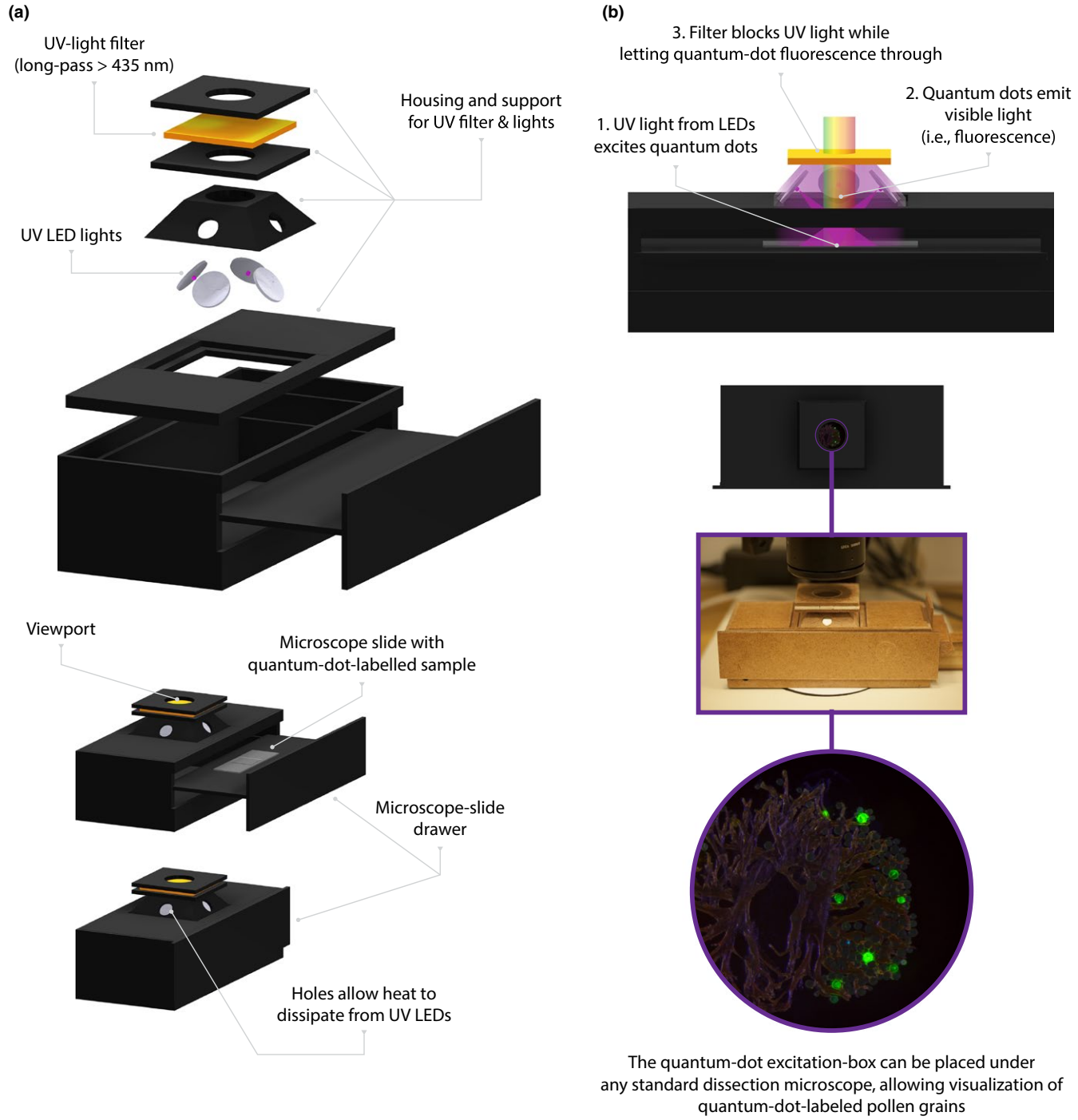

FIGURE 1 Diagram showing the components (a) of the quantum-dot excitation box and how it functions (b). The box contains four LED lights (Intelligent LED Solutions, C3535 1 Powerstar Series UV LED, 390 nm, 400 mW, 125° light angle, 4-Pin) as the UV excitation source. Quantum dots are viewed through a viewport containing a UV blocking, long-pass filter (blocking wavelengths <435 nm; Schott GG435, 50 × 50 mm) which is aligned underneath the microscope objective. The box can hold microscope slides or insects on a drawer that slides in and out of the box. The design presented above has been modified to allow the box to be 3D printed (see Supporting Information for details)

Labnet International, Edison, NJ) and extra-long 10 μl pipette tips (SuperSlik™ 10 μl Extra Long pipette tips; product code—1165-800; Labcon, Petaluma, CA). The very narrow inner diameter of these pipette tips prevents volatile hexane from flowing out of the tip before the quantum-dot solution can be applied to pollen. When applying quantum-dot solution to anthers, we were careful to avoid direct contact between pollen and the pipette tip. We held the pipette tip as close as possible to the upper edge of an anther and ejected the quantum-dot solution slowly onto the anther, allowing it to gently flow across the anther and cover all pollen grains. Hexane is highly volatile (boiling point: 68°C), and therefore evaporates seconds after application, leaving behind quantum dots which putatively bind to the pollenkitt surrounding pollen grains through their lipophilic ligands.

To visualise or 'read' potential quantum-dot pollen labels, we designed and built an inexpensive quantum-dot excitation box that can be placed under any standard dissection microscope allowing visualisation of quantum-dot-labelled pollen on stigmas as well as insects (Figure 1) (see Supporting Information for 3D-printable design files).

2.2 | Method validation

We evaluated Criteria 1 (quantum dots must attach directly to pollen grains) and 2 (quantum dots should label most pollen grains in the anther) using four different plant species from four different families: *Wachendorfia paniculata* (Haemodoraceae), *Sparaxis villosa* (Iridaceae), *Arctotheca calendula* (Asteraceae) and *Oxalis purpurea* (Oxalidaceae). Flowers were collected from urban parks and gardens in Stellenbosch, Western Cape, South Africa. These four species were selected as representative of typical plants to which the quantum-dot labelling technique may be applied. For each species, we determined the appropriate volume for quantum-dot application: we started by applying a 0.1 μl dose of pure hexane to an individual anther and checked whether the volume was sufficient to cover the entire anther. If the volume was too small, we increased the dosage incrementally by 0.05 μl until a single dose was sufficient to cover the entire anther, but not flow beyond anther tissue. Suitable dosage volumes for the four different species were as follows: 0.30 μl per anther for *W. paniculata*; 0.50 μl per anther for *S. villosa*; 0.35 μl per anther for *A. calendula*; and 0.15 μl per anther for *O. purpurea*. This roughly equated to the anther volume (measured as product of the length, height and width of the anther to the closest 0.5 mm) divided by five. Volumes may be increased in hot weather as heat increases evaporation rates of quantum-dot solutions from pipette tips as well as anthers. The effects of heat can also be ameliorated by keeping quantum-dot solutions cold using ice packs in the field.

Next, we determined the appropriate quantum-dot concentration for quantum-dot application to pollen. We initially applied a 2 mg/ml (quantum-dot/hexane) solution at the ideal volume determined for each species. We then placed labelled pollen next to unlabelled pollen on microscope slides using two separate, sterile pipette tips. We examined the grains using the quantum-dot excitation box to check whether labelled grains were easy to distinguish from unlabelled grains. Although labelled grains were distinguishable from unlabelled grains at 2 mg/ml (quantum-dot/hexane) solution, we increased the concentration of the quantum-dot solution to 5 mg/ml for all species to ensure that labelled grains were clearly distinct from unlabelled grains.

2.2.1 | Do quantum dots attach to pollen grains (Criterion 1)?

To test whether quantum dots physically attach to pollen grains, we applied quantum-dot solutions to five different anthers from five different individuals for each of the four species. We then removed the anthers and placed each inside a small centrifuge tube (0.3 ml) containing 100 μl of 70% ethanol–distilled water solution. To dislodge pollen from anthers into the ethanol solution, we vortexed each tube for 2 min. The 70% ethanol solution acts as a polar solvent and will therefore not remove pollenkitt, which is primarily hydrophobic (Pacini & Hesse, 2005), from pollen grains. If dots are physically attached to pollen grains through lipophilic interactions, they should remain on pollen grains when agitated in ethanol. However, if quantum dots are not physically attached to pollen grains, they would likely be removed from pollen grains during agitation in ethanol. After vortexing, we waited 2 min to allow unattached quantum dots to separate from pollen grains suspended within the solution. We then took five 10 μl subsamples of the pollen-ethanol suspension using a micropipette and expelled each subsample onto a separate microscope slide to see if quantum dots remained attached to pollen grains using the quantum-dot excitation box.

2.2.2 | What proportion of grains in an anther are labelled (Criterion 2)?

In addition to confirming quantum dot attachment in the experiment above, we determined the proportion of labelled to unlabelled grains by counting every labelled and unlabelled pollen grain in each 10 μl subsample. We visually counted pollen grains using the quantum-dot excitation box (Figure 1). Quantum dots covered the surfaces of labelled pollen grains causing the entire grain to glow with the colour it had been labelled with, while unlabelled grains appeared dull in comparison (Figure 1). While not all pollen grains present within anthers were counted, we assumed the random sample of pollen grains collected after vortexing was representative of all pollen grains within an anther.

2.2.3 | Do quantum dots influence pollen transport (Criterion 3)?

If quantum dots successfully attach to pollen grains, their presence may still influence how grains are transported, limiting their utility for biologically realistic estimates of pollen movement. To test whether quantum dots influence pollen transport, we conducted multiple pollen transfer trials with labelled and unlabelled *S. villosa* pollen using honey bees *Apis mellifera capensis* as pollen vectors.

Comparing pollen transfer dynamics of labelled and unlabelled pollen under natural conditions is impossible because pollen transferred from target donors cannot be distinguished from pollen already on the vector from previous donors. Therefore, comparisons of labelled and unlabelled pollen transfer require captive vectors that are clean of any pollen.

2.2.4 | Honey bee maintenance and training

We obtained ca. 400 newly emerged adult honey bees from brood frames placed inside an incubator at 36°C for 48 hr. All adult honey bees were removed from brood frames before incubation to ensure that honey bees taken from frames after incubation were newly emerged. We placed the honey bees inside a polystyrene mini-nucleus hive (Apidea: Bruck-enstrasse 6 CH-3005, Bern, Switzerland) containing preformed wax comb with bee bread and a constant supply of 50% sugar solution. The mini-nucleus hive was kept inside a flight cage (70 × 70 × 140 cm) with a central partition dividing the cage into two equal halves. One half of the flight cage housed the bees for training and maintenance, while the other half was reserved for pollen transfer experiments (see below). The flight cage was kept indoors at 25–30°C and a 12:12 hr, light:dark cycle.

Once bees were actively flying within the flight cage (1 week), we trained them to collect nectar from *S. villosa* flowers. To train bees, we placed six emasculated *S. villosa* flowers inside the flight cage for at least 4 hr per day. Each flower was securely attached to the top of 30 cm long bamboo skewers secured to the cage floor. Flower stems were held inside small centrifuge tubes (0.3 ml) containing water. We supplemented nectar (20% w/w sucrose/tap water added to flowers in 5 μl doses) in flowers when empty to ensure that flowers remained rewarding irrespective of honey bee foraging rate. Honey bees foraged consistently from flowers after 3 days of training, at which point we commenced pollen transfer experiments. Training continued throughout experiments.

2.2.5 | Pollen transfer experiments

On the morning of an experimental day, we picked unopened *S. villosa* flowers and randomly split them into two groups, donors and recipients, in a 1:10 ratio. Recipients were emasculated prior to anther dehiscence and used the following day once stigmas were mature and receptive. In addition, we checked the stigmas of all recipients for any pollen grains under a dissection microscope. All flowers with stigmatic pollen were discarded. We removed the stigmas of donor flowers in the morning prior to anther dehiscence and assigned them randomly to one of two treatments: labelled or unlabelled pollen. Once anthers were fully dehisced, we either left the flowers as they were (unlabelled pollen) or applied quantum-dot solution to the anthers as described earlier (labelled pollen).

For each pollen transfer trial, we placed 11 *S. villosa* flowers in a line perpendicular to the cage partition (spaced 5 cm apart) on the experimental side of the flight cage. The first flower in the line acted as the pollen donor, while the next 10 flowers acted as pollen recipients. The donor flower was placed 2 cm away from a small door (5 × 10 cm) in the cage partition. This door could be opened and closed from the outside of the cage, to allow or prevent bees passing from one part of the cage to the other. Flowers were attached to bamboo skewers as before, but we allowed part of the bamboo skewer to extend above flowers which enabled us to cover individual flowers with a small plastic cup to prevent bees from visiting any flower more than once.

To start a pollen transfer trial, we opened the door in front of the donor flower. At the same time, we held a piece of cardboard behind the donor flower so that honey bees could not see the recipient flowers. We waited for a honey bee to fly through the door and visit the donor flower, after which we closed the door and removed the cardboard blocking the recipient flowers. Once the bee finished visiting the donor flower, we covered it with a plastic cup to prevent repeat visitation. Thereafter, we ensured that all flowers received only one visit by covering flowers with a plastic cup (numbered to indicate visit sequence) immediately after being visited. Once a bee finished visiting all of the flowers, we captured and killed it to ensure that pollen was not transported back to the training area. We completed 15 pollen transfer trials for each treatment. Only three trials of 30 resulted in visits to nine recipients instead of all 10 (unlabelled: 2; labelled: 1).

After each trial, we recorded the position in the transfer sequence for each flower and the treatment applied to the donor flower. To account for potential effects of flower morphology on the likelihood of stigma and anther contact with bees, we measured the closest distance between each recipient stigma and the lower lip of the flower (stigma height), and the closest distance between the donor's anthers and the lower lip of the flower (anther height). We harvested the stigmas of each recipient and placed them on individual microscope slides. To prepare stigma slides, we squashed unlabelled stigmas under a cover slip with melted Fuschin gel, while labelled stigmas were squashed under a cover slip without a mounting medium and secured and sealed the edges of the cover slip using transparent sticky tape. Stigma slides were stored at −20°C until pollen could be counted. We counted pollen using a standard dissection microscope with labelled pollen visualised inside the quantum-dot excitation box.

We modelled pollen transfer using a nonlinear, mixed-effects regression of the number of pollen grains deposited on stigmas (pollen count) as an exponential decay function of visit sequence number (visit seq.), stigma height (stigma *h.*) and anther height (anther *h.*):

$$\text{pollen count} = [a + b(\text{anther } h.) + c(\text{stigma } h.)] \times e^{-d(\text{vist seq.})}$$

The expression within the square brackets determines the number of pollen grains deposited on a given stigma for each visit in the sequence. This number is further modified by the expression to the right of the square brackets, which determines the rate of decay (pollen depletion) as a function of visit sequence number. Therefore, a represents an independent intercept, and b and c are expected to be negative because increased stigma and anther heights may result in decreased pollen transfer to bees from the donor flower or due to

poor stigma contact with honey bees. The parameter *d* is expected to be positive and controls the magnitude of pollen transfer decay with increasing visit sequence number. We accounted for potential effects of individual bees on pollen transfer by allowing *a* and *d* to vary for each individual bee (i.e. individual bee ID was included as a random effect on *a* and *d*).

To determine whether treatment had an effect on pollen carryover, parameter estimates for *a* and *d* for each treatment were computed separately and compared using Wald-type *t* tests (Pinheiro & Bates, 2000). Models were computed in r (R Core Team, 2017) using the 'nlme' function (package nlme, Pinheiro, Bates, DebRoy, & Sarkar, 2017).

3 | RESULTS

3.1 | Do quantum dots attach to pollen grains (Criterion 1), and what proportion of grains in an anther are labelled (Criterion 2)?

Quantum dots remained attached to pollen grains even after agitation in 70% ethanol, in all subsamples, for all four species (25 subsamples per species). Moreover, the majority of pollen grains in each subsample were labelled by quantum dots. No unlabelled grains were found in any of the subsamples for *A. calendula*, while nearly all grains were labelled for *W. paniculata* (mean proportion ± *SE*: 0.97 ± 0.01) and *S. villosa* (0.92 ± 0.01) (Figure 2). The proportion of grains labelled for *O. purpurea* was comparatively low, but most grains were still labelled (0.74 ± 0.02).

3.2 | Do quantum dots influence pollen transport (Criterion 3)?

Parameter estimates for *a* and *d* for unlabelled (*a*: $M \pm SE = 876.70 \pm 71.30$; *d*: $M \pm SE = 0.28 \pm 0.030$) and labelled (*a*: $M \pm SE = 886.44 \pm 70.24$; *d*: $M \pm SE = 0.25 \pm 0.024$) treatments did not differ significantly (*a*: $t_{(262)} = -0.60$; $p = 0.55$; *d*: $t_{(262)} = 0.36$; $p = 0.72$). Increased stigma height (*c*) significantly decreased pollen transfer ($M \pm SE = -90.01 \pm 8.48$, $t_{(262)} = -10.61$, $p < 0.0001$), while anther height (*b*) had a similar, but weaker effect on pollen transfer ($M \pm SE = -13.40 \pm 6.82$, $t_{(262)} = -1.97$, $p = 0.0504$). These results indicate that quantum-dot application to anthers of *S. villosa* did not affect pollen carryover (Figure 3).

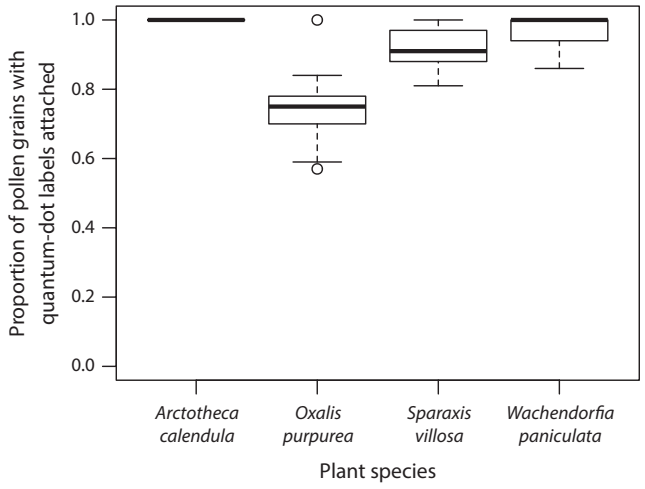

FIGURE 2 Boxplots of the proportion of pollen grains with quantum dots attached after application of quantum-dot solution to anthers of four species and agitation of pollen and anthers in 70% ethanol

4 | DISCUSSION

4.1 | Do quantum dots attach to pollen grains (Criterion 1), and what proportion of grains in an anther are labelled (Criterion 2)?

Our finding that quantum dots remain on pollen grains after agitation in 70% ethanol suggests that quantum dots attached to pollen grains, potentially through a lipophilic interaction between their oleic-acid ligands and the lipid-rich pollenkitt surrounding pollen grains. Nearly all animal-pollinated angiosperms have pollen grains surrounded by pollenkitt (Pacini & Hesse, 2005). Although pollenkitt composition varies, lipids are the primary constituent. Therefore, quantum dots could potentially bind to pollen grains of most animal-pollinated species. A possible exception may be members of Brassicaceae which produce tryphine to coat pollen grains instead of pollenkitt (Pacini & Hesse, 2005). While tryphine is functionally similar to pollenkitt, it is composed of both lipophilic and hydrophilic substances (Dickinson & Lewis, 1973), which may reduce quantum-dot attachment. The exact mechanism of quantum-dot attachment to pollen grains requires further exploration.

Most pollen grains present in anthers were labelled after application of quantum-dot solution for the species tested in our study. However, a lower proportion of pollen grains were labelled in anthers of *O. purpurea*. Anthers of *O. purpurea* release pollen through very narrow apertures that do not fully open upon dehiscence. This semi-closed anther structure may explain why a smaller proportion of *O. purpurea* grains were labelled. It may therefore be difficult to label all pollen grains in species with closed anther structures (e.g. poricidal anthers). However, for most applications, labelling all pollen grains in an anther may be unnecessary, since comparisons of pollen transfer will likely be relative. For studies comparing pollen transfer among species, we recommend quantifying the proportion of pollen grains labelled in anthers following methods in this paper and applying these proportions as species-specific correction factors for quantitative assessments of pollen movement.

4.2 | Do quantum dots influence pollen transport (Criterion 3)?

Pollen transfer experiments revealed no effect of quantum-dot labels on pollen transport for *S. villosa*. This may be a consequence of the small amount of quantum dots required to label almost all pollen grains in anthers. For *S. villosa*, only 2.5 μg of quantum dots was

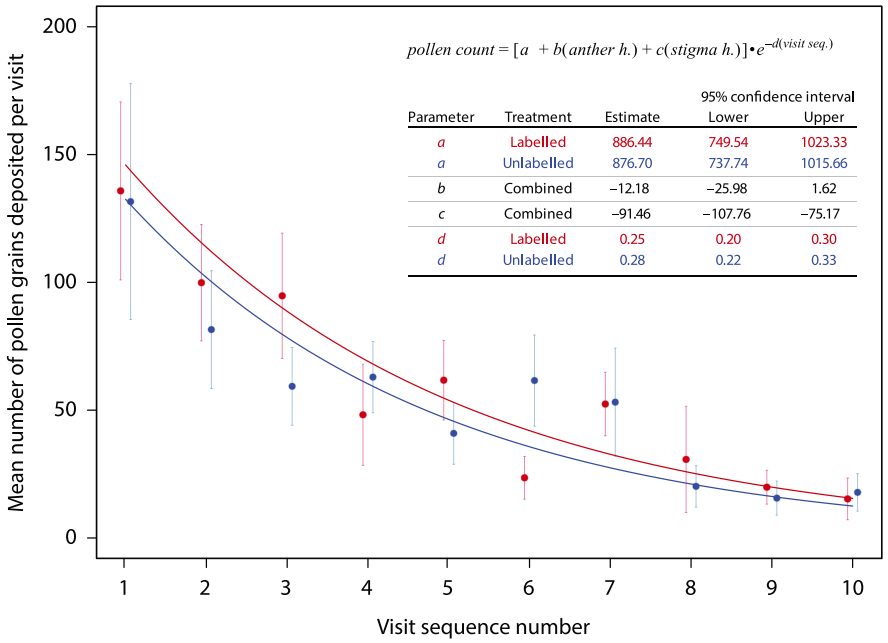

FIGURE 3 The *M* ± *SE* number of unlabelled (blue dots) and labelled (red dots) pollen grains transferred by captive-reared honey bees to a sequence of emasculated *Sparaxis villosa* flowers after a single visit to a *S. villosa* donor flower as a function of visit sequence number. The embedded table shows treatment-specific parameter estimates with 95% CIs from a nonlinear mixed-effects regression model (equation shown above the embedded table) with parameters *a* and *d* estimated separately for labelled and unlabelled pollen. Parameter *a* directly influences the total amount of pollen initially available for transfer, while parameter *d* determines the rate at which the total amount of available pollen is depleted (i.e. transferred to recipients) with each successive visit to recipient flowers in the pollen transfer sequence. Since estimates for *a* and *d* are similar for both treatments and their 95% CIs show a large degree of overlap, it suggests that neither the total amount of pollen available to transfer, nor the rate at which pollen is transferred to recipients is affected by the application of quantum dots

applied to an entire anther. The mass attached to pollen grains is likely even less than that (some quantum dots remain on anthers and in the pipette tip).

Statistically identical intercepts estimated for labelled and unlabelled pollen transfer curves also suggest that the amount of pollen picked up by bees is not affected by quantum-dot labelling. Furthermore, the total amount of pollen transferred by individual bees during trials did not differ between treatments, unlabelled ($M \pm SE$) = 542.33 ± 68.31; labelled ($M \pm SE$) = 581.33 ± 45.63; $t_{(28)} = -0.47$; $p = 0.64$. We conclude that the propensity of pollen grains to attach to bees as well as their propensity to attach to stigmas was unaffected by quantum-dot labelling.

4.3 | Safety and environmental impact

Hexane is highly volatile and evaporates very quickly into surrounding air space when left exposed. Acute short-term exposure to inhaled hexane may cause dizziness, nausea and headaches, while chronic inhalation of hexane fumes may cause damage to the nervous system (Tormoehlen, Tekulve, & Nañagas, 2014). However, the minute volumes of hexane used during application of quantum dots to anthers are unlikely to result in appreciable amounts of hexane being released into the surrounding atmosphere. Exposure to hexane through inhalation may occur during the preparation of quantum-dot solutions when larger volumes of hexane are transferred between containers. However, any potential exposure can be limited by working in a well-ventilated area or inside a fume hood and by closing any open containers directly after use.

Copper–indium quantum dots are often described as non-toxic due to the low risk of toxic effects in humans associated with both metals (Leach & Macdonald, 2016). Nevertheless, long-term environmental effects associated with the release of CuIn quantum dots have not been quantified. While we caution against unreasonably excessive use of quantum dots in field experiments, we doubt the application of quantum dots as pollen labels, even in large field experiments, would result in appreciable environmental exposure to copper or indium. For example, an experiment which requires 500 individual plants to be labelled, each with four anthers (requiring a typical labelling volume of 0.3 µl per anther), would result in <2 mg of CuIn being released into the environment.

4.4 | Limitations, unknowns and future improvements

4.4.1 | Limited colours

Currently, there are only four commercially available, distinguishable quantum dot colours in the visible range. This may limit simultaneous assessments of pollen movement from more than four individual

plants in close proximity. However, there is ongoing research to develop non-toxic colours of short wavelength (blue and violet) and additional colours in the infrared spectrum (up to 950 nm) may be employed as pollen labels in conjunction with infrared-sensitive imaging equipment.

4.4.2 | The effect of quantum-dot labelling on other pollen characteristics

It remains unclear whether brief exposure to hexane, or quantum dots themselves, affects pollen viability. If pollen viability is unaffected, it may be possible to identify individual pollen grains on stigmas as well as their pollen tubes from specific individual plants, and therefore study both pollination and post-pollination components of male reproductive success.

We also did not determine whether pollen grains were structurally, or chemically altered by the quantum-dot labelling process and so it is possible that other aspects of pollination (other than transport) are affected by labelling. For instance, the scent profile of pollen grains may be altered if hexane application draws volatiles out of pollen grains. To the human eye, quantum-dot labelling may change the colour of pollen grains very slightly for some species under visible light and it is unclear how this may affect foraging behaviour of pollinators.

5 | CONCLUSION

Quantum-dot nanotechnology may allow direct assessments of pollen movement in most angiosperms. We anticipate that this will help to quantify (among other things): the magnitude and frequency of pollen loss during various stages of the pollen export process (Inouye et al., 1994); the importance of vector-mediated pollen-movement isolation as a speciation and diversification mechanism in angiosperms (Armbruster, 2014); the structure and competitive implications of the various pollen landscapes that form on vectors as a result of sequential visits to competing conspecifics and heterospecifics (Minnaar et al., 2019); and the importance of specific pollinators in facilitating reproduction and persistence of plants in vulnerable ecosystems and economically important agricultural systems (Potts et al., 2016).

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AUTHORS' CONTRIBUTIONS

C.M. conceived the initial idea for the method and designed the quantum-dot excitation box; C.M. and B.A. jointly developed the method further; C.M. and B.A. designed experiments to validate the method; C.M. collected and analysed the data; C.M. led the writing of the manuscript. Both authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.mc72688 (Minnaar & Anderson, 2019).

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