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*Tools and Technology*

# A Remote Marking Device and Newly Developed Permanent Dyes for Wildlife Research

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**ABSTRACT** Noninvasive, safe, quick marking of individual animals using distinctive colors that are highly visible and persistent is a valuable methodology, but practical techniques and permanent safe dyes are lacking. Here we describe a novel, remotely controlled dye machine to rapidly mark stationary animals in predictable locations, such as birds sitting on nests on the ground or mammals at a den or burrow site. From the month of June when birds were on eggs, using the machine, we spot-dyed 77 California least terns (*Sternula antillarum browni*) at a colony in California, USA, in 4 days without handling them. Concomitantly, we developed a suite of permanent (until molt or shedding), mainly phthalocyanine dyes that are incorporated chemically into feathers or fur of animals and cannot be preened or rubbed off, which have never been used before to dye animals. We found no toxicity of the dyes during *in vivo* testing over 1 month. This method of remote marking with permanent dyes should prove to be a useful method in animal ecology for distinguishing among individuals with minimal disturbance. © 2017 The Wildlife Society.

**KEY WORDS** California least tern, colonial waterbirds, color-marking, marking remotely, permanent dyes, *Sternula antillarum browni*.

There are a handful of field techniques for marking individual animals quickly, with minimal disturbance. Methods to individually mark birds have included colored leg bands in a unique pattern (Marion and Shamis 1977, Baird 1992, Calvo and Furness 1992), colored leg streamers or ptagial tags, and field-readable leg bands (e.g., Marion and Shamis 1977, Lebreton et al. 2003, Nichols et al. 2004). Marking methods for mammals have included tattooing (e.g., Evans and Griffith 1973, Klimisch 1986, McGregor and Jones 2016) and field-readable collars (Moorehouse and Macdonald 2005). Researchers also have used other marking methods to identify individual animals from a distance, including coded radios or dyes, but usually the animals must be trapped to affix the marks or devices.

Hands-off marking techniques would be particularly useful on endangered species. In California, USA, the California least tern (*Sternula antillarum browni*) is designated as Endangered under the 1973 United States Endangered Species Act (as amended; but see Draheim et al. 2010, 2012 for the endangered status). One of this species' largest colonies, distributed over 3 subcolonies, is in southern California at San Diego Bay (Fig. 1). Our research on subcolony-specific foraging behavior needed a long-lasting, highly visible marker because of the difficulty of identifying bands or tags on terns at a distance of >30 m from a survey boat, as well as discerning tagged individuals via telemetry signals, often lost in background electronic noise. We needed marks visible from distance and lasting 2–3 months with no need to remark, and subsequently chose to use dyes because they are an easy and potentially noninvasive way to mark animals (Murray and Fuller 2000) and have been used with varying success on feathers (Marion and Shamis 1977, Calvo and Furness 1992, Donehower and Bird 2005) or fur (Keith et al. 1968, Evans and Griffith 1973, Twigg 1975).

We wanted to minimize human disturbance at the colony; therefore, trapping with hands-on dyeing methods was problematic given long handling times. Hands-on methods used elsewhere include feeding dye to animals, broadcast spraying of animals, and marking eggs or nests. These methods have negative side effects such as toxicity, dyeing nontarget species, or disturbing the colony. We reviewed

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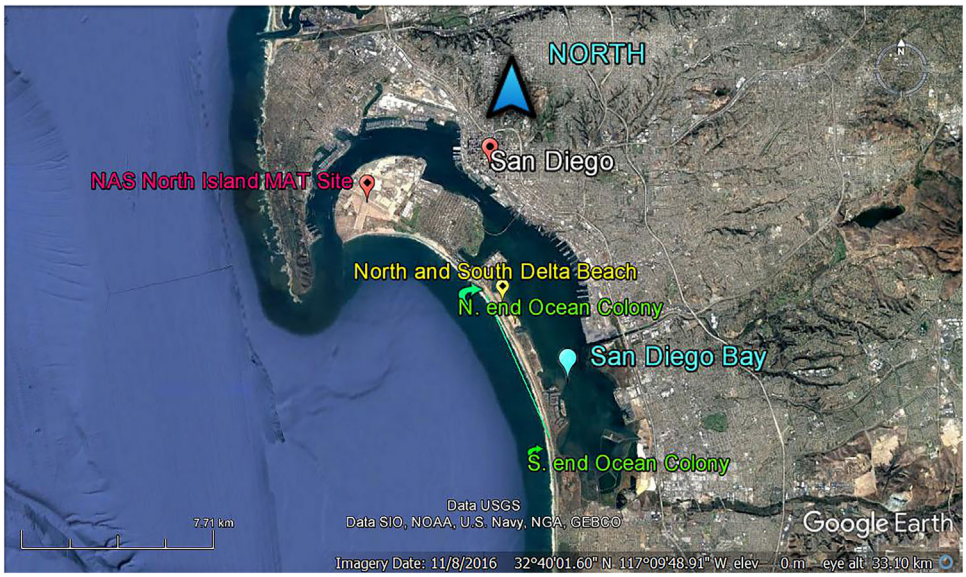


**Figure 1.** Map of the California least tern colonies where we tested dyes and dye-marking equipment in San Diego, California, USA, during 1993–2009. Image source, Google Earth (Google, Inc., Mountain View, CA, USA).

indirect marking methods to find remote methods of dyeing that satisfied conditions of speed, ease, lack of handling, and nondisturbance to the colony (Marion and Shamis 1977, Donehower and Bird 2005; summary of hands-off dyeing methods in Text S1 and Table S1-1, available online in Supporting Information).

One of us (S. A. Hink) developed a compact remote device for dyeing with little colony disturbance, adequate range, accurate aim (a 2–3-cm spot on the breast), and enough force to dye >1 bird at a time. We describe this device, the "dye machine," as well as an experimental dye set developed for longevity, visibility, and safety for use in the machine, which we tested alongside our permitted ("conventional") dyes for visibility and longevity.

## STUDY AREA

We studied California least terns breeding at 3 nesting areas (subcolonies) at the San Diego, California, Naval Base Coronado: North Island colony, Delta Beach colony, and Ocean colony, 1993–1996, 2009 (Fig. 1). One nesting area was in a developed area near a Navy airfield, lightly covered with sand brought from nearby beaches, with low beach vegetation. The second area was on a sand beach facing the Pacific Ocean with sparse low-beach vegetation. The third was an area facing San Diego Bay with packed soil and sparse sand with patchy low-beach vegetation.

## METHODS

### Remotely Controlled Dyeing Machine

*Construction of dye machine.*—We developed a remotely controlled dye machine that could dye several birds at a time. The remote control system (hereafter, RC; Fig. 2) consisted of a transmitter that we disassembled (Airtronics AM transmitter; Fig. 3), a remote distribution unit (Airtronics AV2R receiver and Servo motor; Fig. 4), and up to 4 battery-operated squirt guns (Shout-N-Shoot Squirt Gun Motor and Pump; Fig. 5). The transmitter sent a signal to the radio-activated remote distribution unit filled with dye that was attached to tubes buried in the sand leading to squirt guns pointed at a nest (Fig. 6). A camouflaged tan bowl with sand added to the freshly painted surface hid the squirt gun. The entire system fit into a fishing tackle box (Fig. 7; Table S1-2, Fig. S1-1, S1-2, S1-3).

*Premarking technique.*—Before using the dye machine, we removed all tern eggs from the nest and placed them in a covered bowl filled with bird seed as cushioning, kept in our blind. Then we substituted water-filled speckled plastic eggs resembling real tern eggs in appearance and weight. We exchanged the eggs to prevent damage to the live eggs during the dyeing operation.

We were permitted to use the basic dyes Malachite Green, Methylene Blue, and Rhodamine B (red), and the acid dye Picric Acid (yellow). We purchased all dyes from Sigma Aldrich Corporation, St. Louis, Missouri, USA. We tested these in the dye machine, and determined which 3 of the 4

**Figure 2.** Remote control system of dye machine used to remotely mark California least terns in San Diego, California, USA, during 1993–2009.


**Figure 3.** Disassembled transmitter of dye machine used to remotely mark California least terns in San Diego, California, USA, during 1993–2009.

dyes we would use for the 3 subcolonies. We only dyed adults on eggs that were 7–19 days old (adults are least likely to abandon after 7 days, and the earliest hatching time for eggs was 19 days). Before setting up the equipment, we checked the sites to ensure that we had no target nests with starred eggs ready to hatch (starred eggs means that first visible cracks have appeared).

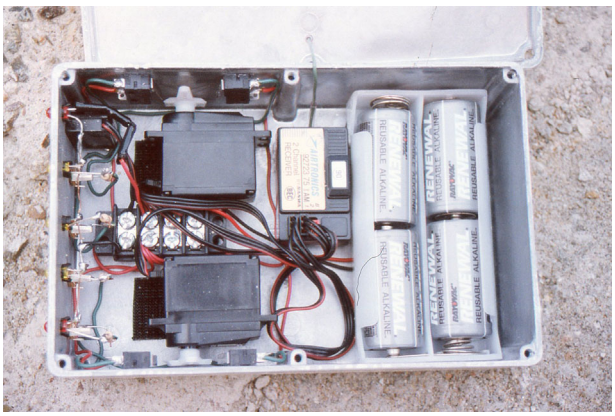

**Figure 4.** Inside of remote distribution unit for dye machine used to remotely mark California least terns in San Diego, California, USA, during 1993–2009.

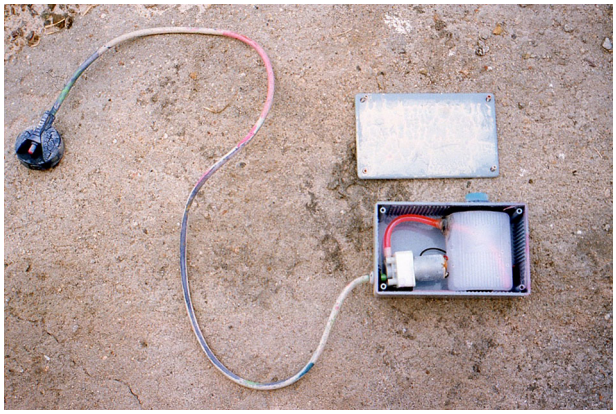

**Figure 5.** Inside of squirt gun in dye machine used to remotely mark California least terns in San Diego, California, USA, during 1993–2009.

*Operation of the dye machine.*—From the blind, we used each transmitter control to send a signal to the receiver in the RC distribution unit, move a switch on the squirt gun, turn on the pump and motor, and squirt dye out the nozzle onto a precise 3–4-cm patch on the bird's breast from a distance of 10 cm (Fig. 8). We could operate up to 4 squirt gun units from 1 RC distribution unit. We determined each bird's preferred incubation position for machine setup, and buried the nozzle with the tip sticking up about 2 cm, and angled slightly up, aimed at the location the bird would normally face when sitting on the eggs. We used 8 dyeing machines at a time. Each transmitter had 2 squirt gun units so that birds on 2 nests could be dyed at a time. We assigned one of the 3 colors (Malachite Green, Rhodamine B, Methylene Blue) to each colony, excluding picric acid because in *a priori* tests, it was too light to be seen at distance. Each dye mixture was saturated in 30% ethanol, and 1 mL Synthrapol or Calsolene (Dharma Trading Compay, San Rafael, CA), for better penetration (Baird et al. 1997). We dyed for 3–4-hr periods to minimize disturbance and did not dye when temperatures were $>29.4^{\circ}$ C or $<12^{\circ}$ C, or when the wind speed was Beaufort >3.

We logged total time that each tern was off-nest from the beginning of setup until its return to incubate on the nest. We allowed each tern 30 min to return before we aborted the session. Once the bird returned and sat facing the nozzle, we

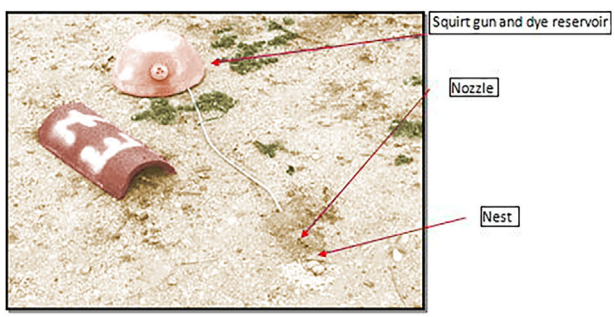


**Figure 6.** Concealed squirt gun of dye machine at nest of California least tern, next to tile shelter in San Diego, California, USA, during 1993–2009.

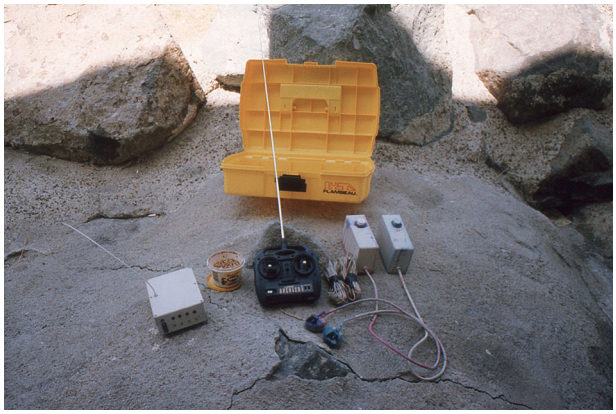

**Figure 7.** Complete dye machine and accompanying field equipment used to remotely mark California least terns in San Diego, California, USA, during 1993–2009.

fired the dye gun. After dyeing, we observed the tern in flight, noting the visibility of the mark. We then retrieved the equipment, checked the final condition of the nest site, removed the plastic eggs, pulled up the nozzle and hose, filled in any surface disturbance, and removed any excess dye from overshoot. Once we restored the site, we replaced the live eggs in the nest. We noted the time until the tern or its mate returned and began incubating again.

### Testing Conventional and Experimental Dyes *In Vitro*

In the *a priori* test we found that, of our permitted dyes, Picric Acid did not wash off from feathers. The dyes that we were permitted to use in the dye machine washed off, so we researched other dyes similar in chemical structure to Picric Acid that have been used on feathers for fly-tying, dyeing cloth, and were known to resist fading when wet. However, because high heat was needed to set them (Talleur 1999, 2010; D. Talleur, freelance author of fly-tying methods and dyes, Clinton Corners, New York, USA, www.dicktalleur.com, personal communication; P. Burch, hand dyeing consultant, Houston, Texas, USA, www.paulaburch.net, personal communication), they were not a suitable method for live birds.

Subsequently, we reviewed dyes used by others, noting species dyed, how dye was applied, dyes used, their visibility, longevity, and toxicity (Table S2, available online in Supporting Information). All dyes had varying success, methods of application varied, and species dyed were diverse: bird species ranged from passerines to seabirds, and mammals ranged from rabbits (Leporidae) to ground squirrels (Sciuridae). Durability of dyes (how long they remained the same color as when they were applied) and their visibility from distances >30 m varied (New 1959, Twigg 1975, Marion and Shamis 1977, Spencer 1978, Savarie et al. 1992). Many of the dyes had solvents that were combustible or toxic (e.g., nyanzol, silver nitrate, picric acid), carcinogenic, or hazardous (e.g., nyanzol, Rhodamine B), otherwise poisonous (e.g., aniline dyes, see Moffitt 1942), or which are systemic and may result in internal marking of tissues (Evans and Griffith 1973, Paton and Pank 1986). We rejected these dyes and sought to find dyes similar in structure to Picric Acid, but that did not need high heat to apply.

With the help of biochemists from Sigma-Aldrich Corporation (Roubal 1997; T. Roubal, Sigma Aldrich, Seattle, Washington, USA, personal communication), a fishing fly-tying business (Talleur 1999, 2010; D. Talleur, personal communication), and an independent textile dye biochemist, (P. Burch, personal communication,), we found 26 other acid dyes or dyes with phthalocyanine, all of which stain amino acids (Table 1). We call these dyes "experimental" because they have never been used to dye animals, although 10 of the 26 are used in food, medicine, or cosmetics.

We set up parallel *in vitro* tests for the 4 permitted ("conventional") dyes and 26 experimental dyes in a 2-month test to select ≥3 dyes for the foraging study. The dyes had to fulfill the following criteria: 1) able to be distinguished from each other through time; 2) require just 1 application; 3) not fade or run; 4) seen easily at a distance of >30 m; 5) last >2 months; 6) adhere to the feathers without having to hold birds until dry; 7) retain their color and adhesiveness after contact with seawater; and 8) nontoxic.

Assuming that dyed birds would bathe in the ocean before the dye dried, we rinsed the feathers in seawater immediately after marking. We used dyes in 30% ethanol because this concentration proved best for longevity, brightness, and visibility at distance (Baird et al. 1997). We made all solutions with nonchlorinated, nonfluorinated distilled water. In the 1997 pilot study, we discovered that adding 1 mL Synthrapol, a detergent and a surfactant, to each 100-mL solution of dye gave better penetration of the feather. Synthrapol solution, a detergent that helps suspend loose dye particles and promotes the best penetration of dye, is used to prescour natural fibers before dyeing, as well as to remove excess dyes during the washing-out process postdyeing (P. Burch, personal communication). The compound has a neutral pH and is gentler on protein fibers (e.g., keratin in feathers) than other detergents. We also found that Calsolene had the same effect by promoting penetration of the dye. This liquid wetting agent breaks the surface tension of the water on the feather and increases the evenness of dyeing, making feathers easier to dye. In our pretrials,

**Figure 8.** Characteristically dyed California least tern following use of a remotely triggered dye machine in San Diego, California, USA, during 1993–2009.

**Table 1.** List of all dyes tested to potentially remotely mark California least terns in San Diego, California, USA, during 1993–2009.[a]

| Common name | Color index name | Molecular formula | Other additives | Color | Comments | Food medicine cosmetics |
|---|---|---|---|---|---|---|
| Acid Orange 63 | Acid Orange 63 | C35H26N6Na2O10S3 | 50% acetic acid | Yellow | Faded | |
| Acid Red 52 | Acid Rhodamine B or Aizen Food Red | C27H29N2NaO7S2 | 50% acetic acid | Red | Light fastness not good | Cosmetics |
| Amido Black 10B | Acid Black 1 | C22H14N6Na2O9S2 | | Did not dye | Needs input of energy (heat) to enter feather (e.g., hair dryer) | |
| Analine blue | | C32H25N3O9S3Na2 | | Did not dye | Dyes connective tissue | Medical research |
| Azo Eosin | Acid Red 4 | C17H13N2NaO5S | 10% acetic or citric acid | Bright red | | Food, cosmetics |
| Cobalt phthalocyanine | | C32H16CoN8 | | Even blue-black | | Medical use |
| Coomasie G250 | Acid Blue 90 | C47H48N3NaO7S2 | | Blue | | Medical use |
| Coomassie R | Acid Blue 83 | C45H44N3NaO7S2 | | Blue | | Medical use |
| Copper Phthalocyanine tetrasulfonic acid | Acid Blue 249 | C32H12CuN8Na4O12S4 | 10% calsolene | Bright Blue | | Medical use, Cosmetics, Tattoos |
| Lissamine Green (Disodium Phthalocyanine) | Acid Green 50 | C27H25N2NaO7S2 | 10% calsolene | Even dark blue | | Medical use |
| Eosin Y | Acid Red 87 | C20H6BrNa2O5 | Added polar solvent (DMSO) | Red | Faded | |
| Erioglaucine (FD&C 1) | Acid Blue 9 | C37H34N2Na2O9S3 | | Did not dye | | |
| Fast Green FCF | Food Green 3 | C37H34N2Na2O10S3 | | Did not dye | | |
| Indigo Carmine | Acid Blue 74 | C16H8N2Na2o8s2 | | Did not dye | | |
| Iron (III) phthalocyanine | | C32H16FeN8 | 10% citric acid | Dark black | | Medical use |
| Magnesium phthalocyanine | | C32H16MgN8 | 10% calsolene | Dark teal | Rinsed out- precipitated out | |
| Malachite Green | Basic Green 4 | C23H25ClN2 | Base | Green | Rinsed out | |
| Manganese (II) Phthalocyanine | | C32H18MnN8 | 10% calsolene | Medium black | | Medical use |
| Methyl Blue | Acid Blue 93 | C37H27N3Na2O9S3 | | Did not dye | | |
| Methyl Orange | Acid Orange 52 | C14H14N3NaO3S | | Did not dye | | |
| Methylene Blue | Basic Blue 9 | C16H18ClN3S | Base | Blue | Rinsed out | |
| Nigrosin (Acid dye) | | C22H16N603 | | Did not dye | | |
| Ninhydrin Purple | | C9H6O4 | | Did not dye | Dyes proteins–amines | |
| Orange G | Acid Orange 10 | C16H10N2Na207S2 | | | Did not dye | |
| Picric Acid | | C6H3N3O7 | Acid | Yellow | Very light | |
| Remazol Brilliant Blue R | Reactive Blue 19 | C22H18N2O11Na2S3 | | Did not dye | | Medical use |
| Rhodamine B | Basic Violet 10 | C28H31ClN2O3 | Base | Violet-red | Rinsed out | |
| Tartrazine | Acid Yellow 23 | C16H9N4Na3O9S2 | | Yellow | Rinsed out | |
| Zinc phthalocyanine | | C32H16N8Zn | | Light teal | Rinsed out and precipitated | Medical use |

[a] All dyes purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA; www.sigmaaldrich.com/).

some dyes proved to be brighter with the addition of Calsolene HSO, which acidifies the solution.

Our standardized tests for all 30 dyes used a solution of 30% ethanol and 1% Synthrapol or Calsolene on 2 kinds of feathers: 1) feathers from a pillow, and 2) naturally molted contour feathers. We acquired the first set of molted feathers from dropped gull (Laridae) feathers found on the beach. Later, we acquired feathers from black-necked swans (*Cygnus melancoryphus*), donated by the Greater Los Angeles Zoo (M. Hines, Curator). The latter proved the best testing feathers in part because they were large, undamaged, and free of sand. Pillow feathers had been bleached and treated so, to compensate, we simulated natural oils by applying canola oil. To test the effects of the oil on dye penetration, we left half of the feathers unoiled. We did not oil the gull or swan feathers because they had natural oils. To simulate effects of immersion in the ocean after being marked, we washed dyed feathers by dipping and swirling in seawater. To simulate the birds' preening, we wrung dyed feathers with moderate pressure between index finger and thumb 3 times.

We set up time increments of 5, 10, 15, and 30 min after dyeing to wash and wring feathers. Also, we included a subset of feathers dried with a hand-held hair dryer before washing and wringing for a control. One hour after the initial rinsing treatments, we put all feathers in the sun, and washed and wrung them for 2 min every 30 min for 3 hr, simulating immersion in the ocean and preening. We left all feathers in the sun for 2 months and observed them every 2 weeks, from 10, 20, and 30 m, noting color quality and detectability at each distance. On completion, we ranked all of the dyed feathers qualitatively based on the amount of fading that had taken place over the course of the initial tests and at what distance we could see the colors well. We did not use scientific instruments to measure visibility (e.g., reflectance measured by a spectrophotometer) because we wanted data showing qualitative values based on a practical human determination of visibility.

### Testing Experimental Dyes *In Vivo*

The goal was to use these dyes on endangered California least terns, so we first tested them on a surrogate species, brown-headed cowbirds (*Molothrus ater*; hereafter, cowbirds; mass 38–50 g), a common bird similar in mass to least terns (30–45 g; Cornell Laboratory of Ornithology 2015). We trapped and then banded 35 free-living cowbirds with U.S. Geological Survey Bird Banding Laboratory (USGS) bands and uniquely colored plastic leg bands and kept them in a flight cage 4.57-m (15 ft) × 3-m (10 ft) × 2.44-m (8 ft) in height, equipped with dowels to perch on. We provisioned water and birdseed daily. Cowbirds were free to fly short distances and forage on the ground.

We chose 6 dyes from the 26 dyes from Sigma Aldrich (St. Louis, MO, USA), which we tested in the *in vitro* trials, to use on the surrogates, because they were approved by the U.S. Food and Drug Administration for use in food, cosmetics, or medicine: Azo Eosin (Acid Red 4), Copper Phthalocyanine (Acid Blue 249), Lissamine Green (Acid Green 50), Manganese (II) Phthalocyanine, Cobalt Phthalocyanine, and Iron (II) Phthalocyanine. Most compounds toxic to mammals are also toxic to birds (Dumonceaux and Harrison 1997); therefore, an *in vivo* test on cowbirds (as examples of all birds) would suffice for one on mammals, and *vice versa*. We divided the 35 birds into 7 groups of 5 birds each: 1 group for each experimental dye tested and 1 control group that we painted with isopropyl alcohol and Synthrapol.

Dyes painted on birds were dissolved in the same concentration as dyes used on the *in vitro-tested* feathers (30% isopropyl alcohol with 1 mL Synthrapol). For Copper Phthalocyanine, Lissamine Green, and Manganese (II) Phthalocyanine, we added 10% Calsolene for better wetting. For Azo Eosin, we added 10% citric acid for better penetration. We painted dyes onto the scapulars, tail, and right foot of each bird with a foam brush. We held birds until the dye dried—<5 min. We monitored birds for 30 days, and observed each bird for 10 min twice per day at randomly assigned times, to determine if they were active and eating. We scored birds in the same way we had scored them during the acclimatization period.

We monitored for signs of listlessness, imbalance, or other gross indicators of ill health to assess whether there was an acute poisoning or adverse effect from the dyes. Also, we looked for specific signs of photophobia, epiphora (tearing), coughing, sneezing, hyperventilation, shortness of breath, somnolence, salivation, bloody feces, or vomiting, which are acute markers of toxins in the blood (Dumonceaux and Harrison 1997). Scores were on a qualitative scale of 1–3 for each 10-min period of observation, with 3 being highest, for a) activity level (1 = listless, 2 = inactive, 3 = active), b) eating (1 = did not eat, 2 = tried to eat but could not, 3 = ate), c) apparent health (1 = wings droopy or eyes closed, 2 = feathers not preened or eyes irritated, 3 = normal appearance). We did not measure seed or water consumption, nor weigh birds. We dosed these birds only once with dye, so we expected no long-term or chronic effects. At the end of 30 days, we released all birds.

We followed protocol on permits from the U.S. Fish and Wildlife Service Endangered Species Office, the USGS Bird-Banding Permits and on the Memorandum of Understanding from the State of California. During all research, we adhered to the Ornithological Council's trapping and marking guidelines for birds (Fair et al. 2010).

## RESULTS

### Testing Conventional and Experimental Dyes *In Vitro*

Pillow feathers did not hold the conventional dye well. All basic dyes tested with washing increments of 5 and 10 min washed out of all sets of feathers (molted and treated). All dyed feathers from the 15-min washing increment faded considerably, so that they were hard to see at a distance of >10 m. Only molted feathers dried with a hair dryer or washed after 30 min were visible at a distance of ≥20 m after washing. Even for these, all of the conventional basic dyes that had not rinsed out immediately faded during the 3-hr postdyeing washing regime and lasted no longer than 2 weeks; then were only visible at <10 m. Picric Acid did not

**Table 2.** Success rates of marking and percentage of remotely dye-marked California least terns in San Diego, California, USA, during 1993–2009.

| Colony | Population | No. of attempts | Attempts: % of population | No. of hits | Success rate: hits/attempts | Dyed population |
|---|---|---|---|---|---|---|
| Delta | 218 | 20 | 9.17% | 18 | 90.0% | 8.26% |
| North Island | 48 | 30 | 62.5% | 16 | 53.33% | 33.33% |
| Ocean | 76 | 54 | 71.05% | 43 | 79.63% | 56.58% |
| Total | 342 | 104 | 30.4% | 77 | 74.04% | 22.51% |

fade up to 2 months postdyeing, but its color was too light to be visible at a distance of >10 m.

Colors of the 6 chosen experimental dyes did not rinse out, and were visible from ≥30 m over >2 months without fading. These 6 were 1) the sulfonated derivative of Copper Phthalocyanine (Copper Tetrasulfonic acid, Color Index Acid Blue 249), 2) Azo Eosin (Color Index Acid Red 4), 3) Lissamine Green B (Color Index Acid Green 50), 4) Cobalt Phthalocyanine, 5) Manganese Phthalocyanine, and 6) Iron (II) Phthalocyanine.

### Development of Dye Machine Using Conventional Dyes

The dye machine marked terns with minimal disturbance, and all dyed birds returned to incubate their eggs. Operating the machine improved with experience, and the "colony success rate" (birds dyed per attempt), increased from the first colony where we dyed, North Island, to the last, Delta colony (Table 2). Subcolonies differed in numbers of nesting pairs (48 North Island, 76 Ocean, 218 Delta); thus, our success rate (% of dyed birds/colony) depended both on our technique and colony size.The overall success of dyed birds per dye attempt was 74%. We dyed a mean of 22.5% of all nesting birds on the 3 colonies [range = 8.3–56.6%]).

We labeled a dye shoot unsuccessful if the tern remained off-nest for >30 min before we could attempt to dye or if it sat in an incorrect position for aiming the nozzle for >30 min. We divided off-nest times into 1) time from when we first flushed the adult from the nest while we set up the dye machine until it resettled on the dummy eggs, and 2) time from when the adult left the nest postmarking until it again settled on the real eggs. The average total off-egg time before marking was greater than after marking, but it was well within the 30 min we had set as a maximum for being off-nest (Table 3). In 96 dye bouts, the mean minutes we spent on-site (setup, in the blind waiting for return, and removal of the machine) were $22.7 \pm 12.3$, and median minutes onsite was 20.0. During our setup activities, terns generally flew above or stood within 6–8 m of the nest, watching. Our time onsite and time of terns off the nest differ because we often dyed >1 tern simultaneously, using ≥2 machines at once.

All dyed birds went to the water immediately after being dyed, but soon returned to their nests. Terns plunge-dive and constantly wet their feathers, so the conventional dyes we used washed off after 2 days and we could not distinguish birds from different colonies.

### Testing Experimental Dyes *In Vivo*

The acid and metal phthalocyanine dyes that we wanted to use in the dye machine had never been tested on live animals. Although they were not as visible on the brown feathers as they were on white feathers in our *in vitro* tests, testing on cowbirds of the same mass as least terns sufficed for testing toxicity. None of the birds in any test group (including the control group) showed any signs of ill health or inactivity. No birds scored a 1 (listless) or 2 (inactive) on the 3 measures of: activity, eating, and general health. Two birds ingested the dye by biting the brush as the dye was applied (Acid Blue 249 and Iron (II) Phthalocyanine). Neither of these showed any ill effects over the 30 days held. The activity, eating habits, and health of dyed birds were the same as undyed birds. All birds had a score of 3 for all periods of observation in the categories activity, eating, and apparent health. All birds had a score of 3; therefore, we did not perform test statistics.

## DISCUSSION

### Development of a Remotely Controlled Dye Machine

Our remotely activated dye machine was an improvement on previously used remote marking units and superior to other methods for marking birds without handling them. The squirt gun provided an efficient and nonintrusive method of marking, and we recommend the dye machine for marking large numbers of ground-nesting birds, or mammals that have regular lookout sites or areas in front of their dens or burrows. The technique is especially effective for marking colonial ground-nesters because many birds can be dyed simultaneously.

Previous devices that squirted dye were not suitable for targeting individuals because of inaccurate dye application (e.g., broad-swath sprinklers or throwing dye-filled objects at birds), or faults in the equipment (e.g., cumbersome equipment with disturbance issues, or by low pressure at the output; Moffitt 1942, Tickell 1968, Moseley and Mueller 1975, Wendeln et al. 1996). Passive methods such as dyeing

**Table 3.** Mean (±SE) minutes of off-egg times of California least terns before and after being remotely dye-marked in San Diego, California, USA, during 1993–2009.

| Off-egg | Before marked[a] | After marked[b] | Total |
|---|---|---|---|
| *n* | 101 | 80 | 181 |
| Mean min. | 19.03 ± 1.00 | 13.83 ± 1.39 | 32.41 ± 2.02 |
| Median min. | 18 | 9 | 29 |

[a] Before marked: From when the adult was first flushed from the nest while we set up the apparatus until it resettled on the dummy eggs once we were in the blind.

[b] After marked: When the adult left the nest postmarking until it again settled on the nest.

eggs or placing dye in front of or in nests potentially jeopardizes live eggs (Mossman 1960, Paton and Pank 1986, Cavanagh et al. 1992, Belant and Seamans 1993, Donehower and Bird 2005). Donehower and Bird (2005) state likewise that the technique of self-marking by birds where dye is placed in the nest is not good because the pattern of dye is more random and individuals cannot be marked at a targeted part of their bodies. The same is true for techniques such as paintball land mines Fox (2010) and eggshells or light bulbs thrown at birds (Bendell and Fowle 1950).

The physical layout of the North Island colony contributed to the low success rate of dyeing. This colony was located on a concrete airfield with a thin layer of sand on top, which caused a problem in the placement and concealment of the nozzle and hose. Concealment of the hose helps to keep it in place, but on the concrete airfield with little ground cover or sand, the hose could move slightly with a light breeze or from the force of the dye going through it; because of this, the aim of the hose there was not perfect. However, despite these factors, the overall success rate of the dye machine on all 3 subcolonies (hits/attempts) was 74%; excluding North Island, the success rate was 82%.

Most of the unsuccessful attempts after the first week were due to the terns' positions once on the nest and not problems with the gun. Wendeln et al. (1996) encountered similar problems with differences between bird orientation and nozzle direction making dyeing impossible. Lack of success was also not from an adult being off-nest during the dye session for >30 min. Terns did not react to the presence of our equipment or us, once we returned to the blinds.

The entire process did not appear to have negative effects on the birds. All birds dyed hatched the eggs on which they had been sitting when dyed. We did not observe any increase in frequency of predation on dyed birds. Average total off-egg time both before and after marking was small, suggesting minimal disturbance to the terns. We did not have data on hatch success of other terns that we did not dye.

### Testing Conventional and Experimental Dyes *In Vitro*

We fulfilled our goal to find dyes that lasted ≥2 months and were visible from >30 m without having to be dried after application; these were either acid or phthalocyanine "experimental" dyes. Most dyes used in prior studies on birds or mammals faded and were difficult to detect at distance depending on how much of the animal was dyed (Kozlik et al. 1959), or how saturated the dye became (M. Haramis, U.S. Geological Survey, personal communication; Table S2, available online in Supporting Information). Depending on methods of application and concentrations of solutions, some dyes lasted no more than 2 weeks (Cavanagh et al. 1992, Belant and Seamans 1993), although Evans and Griffith (1973) used Rhodamine B, Malachite Green, or Picric Acid, which lasted 4 days to 5 months, depending on application method and dye. The success of basic dyes for >2 weeks on most animals was because either the animals were held until drying of the dyed feathers (Kozlik et al. 1959, Moseley and Mueller 1975, Warnock et al. 1995, Donehower and Bird 2005) or dyed fur (Evans and Holdenried 1943, Keith et al. 1968, Evans and Griffith 1973), or because of the large amount of dye placed on the animal. Evans and Griffith (1973) placed so much dye on rabbits, that they found dye internally, dyeing organs, and Forster (1973) put so much dye on swans that they could not fly. Commercial fur dyes lasted a median of 30–60 days on Beechey ground squirrels (*Otospermophilus beecheyi*; Evans and Holdenried 1943), although today, the dyes are considered hazardous, explosive, and unsuitable for use on live animals. Dyes that lasted the longest without fading ranged from a maximum of 3 weeks for Belant and Seamans (1993), 28–42 days for Cavanagh et al. (1992), to 4.5 months for Furness and Galbraith (1980).

Others have used Batik dye or permanent markers, which we determined unsuitable for our use because of their harmful ingredients and their impermanence (Wadkins 1948, Kennard 1961, Donehower and Bird 2005). Kennard (1961) used Drimark markers applied via a with a sponge, on black-capped chickadees (*Poecile atricapillus*), and only the red Drimark lasted >4 weeks. However, ingredients in Drimark dyes might be unsafe because they contain toxic ingredients (commercial dye in a highly volatile organic carrier solvent, methyl isobutyl ketone–n-butyl acetate, diethylene glycol monobutyl ether–ethyl acetate–poly ethylene glycol, or polycarboxylic acid, and has metallic dye particles suspended in a resin polymer, Sukhna and Reichmann 2003). Vegetable and commercial wool dyes, and stamp-pad ink did not last for >2 weeks (Kennard [1961]); and ink-jet printer ink ran when in contact with water (Fox 2010). The ink is also basic and thus similar to textile dyes (Nyman and Hakala 2011).

Of the 26 experimental dyes we tested, 10 proved to be the best in longevity and detectability among other acid and basic conventional dyes, and 10 were used in medicine or in the cosmetic or food industry of the United States and approved as safe by the U.S. Food and Drug Administration. Of dyes approved as safe, only 8 had good longevity and detectability. We were left with 6 good dyes because Coomasie G250 and Coomasie R were too expensive. Copper Phthalocyanine Tetrasulfonic acid–Acid Blue 249 is used to color polypropylene nonabsorbable sutures for use in general and ophthalmic surgery (Food and Drug Administration 2010, pubMed.gov 2014) and in tattoos (Kaur 2016). Azo eosin–Acid Red 4 is used to inhibit toxins of fungus in food (Hitokoto et al. 1980) and added to nail polish (Sharma and Kaur 1994) and lipstick (Anstead 1959). Lissamine Green–Acid Green 50 is used in ophthalmological surgery or diagnostics (Manning et al. 1995, Kim 2000, Abelson and Ingerman 2005, Hamrah et al. 2011), and Manganese (II) Phthalocyanine is used in photodynamic therapy for treatment of cancer (Moreira et al. 2008). Cobalt Phthalocyanine and Iron (II) Phthalocyanine are in a class of drugs that is pharmacologically inactive, but when exposed to ultraviolet radiation or sunlight is converted to its active metabolite that affects diseased tissue (Staicu et al. 2013). These compounds can be administered topically or systemically and have been used therapeutically to treat psoriasis and various types of neoplasms as well as

Alzheimer's disease (Pankratov et al. 2014, Tabassum et al. 2015). Cobalt Phthalocyanine is also an antitumor delivery system by itself when combined with citric acid (Medvedev and Leshchenko 2012*)*. The phthalocyanines themselves seem to have useful properties for cancer therapy (Ben-Hur and Rosenthal 1988).

Using this suite of acid and phthalocyanine dyes in the dye machine would be ideal for marking large numbers of colonial seabirds or mammals that are stationary in predictable places. There are permit restrictions on handling endangered species, so the dye machine would be an easy hands-off way for marking endangered colonial birds or endangered mammals. Paired with the permanent dyes, using the dye machine would also be a way to deliver dye to animals that researchers do not want to trap in order for the dye to dry. We define "permanent" as not fading and keeping a true color until molt or shed of feathers or fur.

### Biochemical Differences between Acid (Experimental) and Basic (Conventional) Dyes

The superiority and permanence of acid or metal phthalocyanine dyes compared with basic dyes lies in the molecular structure of the dye itself, not of the wetting compound. Unlike basic dyes that are topical, acid dyes and metal phthalocyanine dyes have the ability to form hydrogen bonds with the amino acid groups on the keratin protein, which makes up the majority of feathers or fur. Thus, they are incorporated into the feathers or fur themselves. The 2 also attract each other by van der Waals forces (P. Burch, personal communication). Acid dyes typically have a colorless cation such as sodium, and a colored anion. Amino acids on feathers or fur have a positive charge so that the negatively charged anion of dye binds to them.

Basic dyes have a colored cation, such as Methylene Blue, combined with a colorless anion, such as chloride. The negatively charged and colorless anion of chloride will not cause a noticeable color change when bonded to the positive amino acids of the fur or feathers. When rinsed, the colored positive cation will wash away because it is not incorporated into the feather (P. Burch, personal communication; Talleur 2010; D. Talleur, personal communication).

Colors of basic dyes attach to the feather only when bound to an adherent such as silica gel so that they sit on top of the feather, temporarily sticking to it (Belant and Seamans 1993, Talleur 2010, D. Talleur, personal communication). The colored portion is never incorporated into the feather, and essentially is glued to the surface of the feather (summary of biochemistry of acid dyes in Supplement S3, available online in Supporting Information).

Dyes thus incorporated into feathers or fur are unable to be ingested or preened off and, therefore, are particularly suited for use in wild animals as harmless dyes. In contrast, basic dyes are ingestible because they are only physically and topically adhered to the top of feathers or fur via resins or gels, and thus potentially more toxic than are acid or phthalocyanine dyes. Any choice of acid or phthalocyanine dyes should be limited to those used in either foods for human consumption, in cosmetics, or in medical testing. Dyes other than these should be tested on live animals for toxicity.

### Testing of Experimental Dyes *In Vivo* on a Surrogate Species

We showed that the 5 experimental dyes were not harmful to brown headed cowbirds over a 30-day period postdyeing. The subjects showed no signs of photophobia, epiphora (tearing), coughing, sneezing, hyperventilation, shortness of breath, somnolence, salivation, bloody feces, or vomiting, which are symptoms of toxins (Dumonceaux and Harrison 1997). It was beyond the scope of this project to analyze any blood markers for toxins. The dyes did not mat the feathers because they dried quickly; there was such a small amount of liquid squirted onto the feathers that we presume the dye did not change the thermal properties of the feathers.

Advantages of using acid and phthalocyanine dyes are 1) permanence of color until molt in birds or shedding in mammals, 2) no change in observed color over time, and 3) inability of an animal to ingest the dye because it chemically binds with the feather or fur on contact. The experimental dyes would be ideal to mark species that contact water. Conventional dyes (basic dyes, indelible ink markers, Batik, etc.) have the disadvantages of fading quickly, nonadherence to feathers or fur, and potentially toxic carriers able to be ingested because they are not chemically incorporated into the animal's feather or fur. Conventional dyes also have a wide range in durability or in visibility from >30 m, and most colors fade quickly over time.

Some acid or metal phthalocyanine dyes hold promise for permanent marking of animals because these experimental dyes seemed to have had no adverse effects on cowbirds, and so the next step for an approval of this suite of dyes by the U.S. Geological Survey Bird Banding Laboratory and the U.S. Fish and Wildlife Service would be to apply these dyes to other birds such as seabirds or ducks (Anatidae) and test the *in vivo* permanence of the dyes in conditions where the bird is exposed to water. They could also be applied to mammals that use water, although the majority of these have dark fur and the dye probably could not be detected.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

**Supplement S1**. Detailed methods of dye machine with tables, figures, references.

**Table S1-1**. Marking techniques used in other studies.

**Table S1-2**. Parts list of the remote dye applicator ("squirt gun").

**Table S1-3**. Parts list key to distribution unit.

**Figure S1-1**. Remote dye applicator "squirt gun."

**Figure S1-2**. Top view of remote distribution unit.

**Figure S1-3**. Front view of remote distribution unit.

**Supplement S2**. Table of summary of other studies on dyeing birds, including species, dyes used, longevity, toxicity, duration, references.

**Supplement S3**. Biochemistry of dyes. Summary and references.