Intropica 18 (2): 153-182

Generation of high-resolution digital images of arthropods: solutions designed for Latin American collections

Generación de imágenes digitales de alta resolución de artrópodos: soluciones pensadas para colecciones en Latinoamérica

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Abstract

Biological collections are important reservoirs of biological heritage. As such, these institutions constantly try to preserve and study the specimens in their custody and make the information accompanying them available. Scientific collections have benefited from recent technological advances that make an enormous capacity available for generating images that facilitate the achievement of preservation and dissemination objectives. However, generating high-resolution images is seen as a process with a high demand for equipment and an elevated budget investment, hampering its implementation. For this reason, in this work, we synthesize the requirements and steps necessary to obtain, process, and store arthropod images, proposing solutions for different costs that can be adjusted to the needs and realities of Latin American entomological collections and their potential users.

Key words: photography; biological collections; digitization; specimens

Resumen

Las colecciones biológicas son importantes reservorios del patrimonio biológico. Estas instituciones desarrollan esfuerzos constantes para preservar y estudiar los especímenes que custodian, así como para hacer disponible la información que acompaña a dichos ejemplares. Los avances tecnológicos recientes les han brindado a las colecciones científicas una enorme capacidad para producir imágenes que facilitan alcanzar los objetivos de preservación y divulgación. Sin embargo, la generación de imágenes de alta resolución es vista como un proceso con alta demanda de equipos y elevada inversión presupuestal, lo que dificulta su implementación. Por ello, en este trabajo se sintetizan los requerimientos y los pasos necesarios para obtener, procesar y almacenar imágenes de artrópodos, proponiendo soluciones de diferentes costos que pueden ajustarse a las necesidades y realidades de las colecciones entomológicas latinoamericanas y de sus potenciales usuarios.

Palabras clave: fotografía; colecciones biológicas; digitalización; especímenes

***Autor de correspondencia:** cxf5395@psu.edu
Editor: Juan Carlos Narváez Barandica
Recibido: 23 de junio de 2023
Aceptado: 10 de julio de 2023
Publicación en línea: 30 de diciembre de 2023
Citar como: Flórez V, C., Cardona-Duque J. y Jiménez-Ferbans, L. (2023). Generation of high-resolution digital images of arthropods: solutions designed for Latin American collections. *Intropica*, 18 (2), 153-182. https://doi.org/10.21676/23897864.5362.

Introduction

Through decades and even centuries of continuous work, biological collections have become institutions that house a historically and scientifically significant sample of biological heritage (Holovachov *et al.*, 2014). Due to current dynamics and societal demands, preserving, studying, and providing access to this repository of information have become the most important goals for museums and biological collections (Brecko and Mathys, 2020). To this end, processes and tools have been continually incorporated to facilitate managing, preserving, and handling biological material.

However, preserving, cataloging, systematizing, and subsequently presenting and disseminating the information stored in collections is not easy, especially considering the volume of stored material. For example, in Colombia, there are 290 biological collections officially registered with government entities (National Collection Registry: http://rnc.humboldt.org.co), which house over 60 million specimens (Colombian Biodiversity Information System: SiB Colombia). Most of these are invertebrates, of which only 12.2 % are cataloged, and about 10 % are openly published through SiB Colombia (Alexander von Humboldt Biological Resources Research Institute [IAvH] and Ministry of Environment and Sustainable Development 2023: RNC in Figures).

Consequently, in addition to the effort required for cataloging and systematizing, digitalization solutions must be implemented, including label data and specimens (i.e., photographs). This approach allows for optimizing the process and developing strategies for efficient information exchange with local specialists and experts worldwide, decision-making entities, and the general public.

Recent technological advances have provided scientific collections with a tremendous capacity to produce images, making them essential tools for supporting a wide range of research. Thus, few technologies are as prevalent in modern biological laboratories as those focused on generating these visual resources (Eliceiri *et al.*, 2012). Among biological collections, some uses for photography are referencing material type, promoting remote consultation (avoiding unnecessary shipments that expose the material to transport damage), facilitating taxonomic identification, and supporting taxonomic studies (e.g., consultation of morphological characters and morphometric analyses).

One of the limitations of generating high-quality photographs of arthropods due to their small size is the depth of field. Typically, a single photograph cannot entirely focus on the specimen, which is why the focus stacking technique has been developed. In this method, multiple photographs are taken, each with a different focal depth (Figure 1A), and these are combined (stacked) into a final image (stacked image) (Figures 1B, 1C, 2) using a program that detects the focused areas of each photo. This technique, along with proper lighting, offers many advantages, as it allows for highly detailed images of the different structures of the organisms.

The equipment and methods required to implement focus stacking are pretty varied, so several aspects must be considered when acquiring and using them to obtain images with specific quality and availability requirements (Brecko and Mathys, 2020). Therefore, collections should establish protocols to obtain photographs, ensuring information transmission and meeting user demands. Considering requirements and budget, these protocols should include necessary equipment, a selection of material to be digitized, the steps and considerations for image capture (including specimen selection and preparation, lighting types, software for camera control and image stacking), image editing, dissemination, and file management.

In many cases, the implementation of focus stacking in collections is limited by budgetary constraints. However, this technique may require a much smaller investment than initially thought. Although many collections have the basic equipment to generate high-resolution images, they often are unaware of its utility and the simplicity of additional requirements. Therefore, this work synthesizes the requirements and steps necessary to obtain, process, and store arthropod images using focus stacking while proposing solutions of varying costs that can be adapted to the needs and realities of Latin American entomological collections and their potential users.

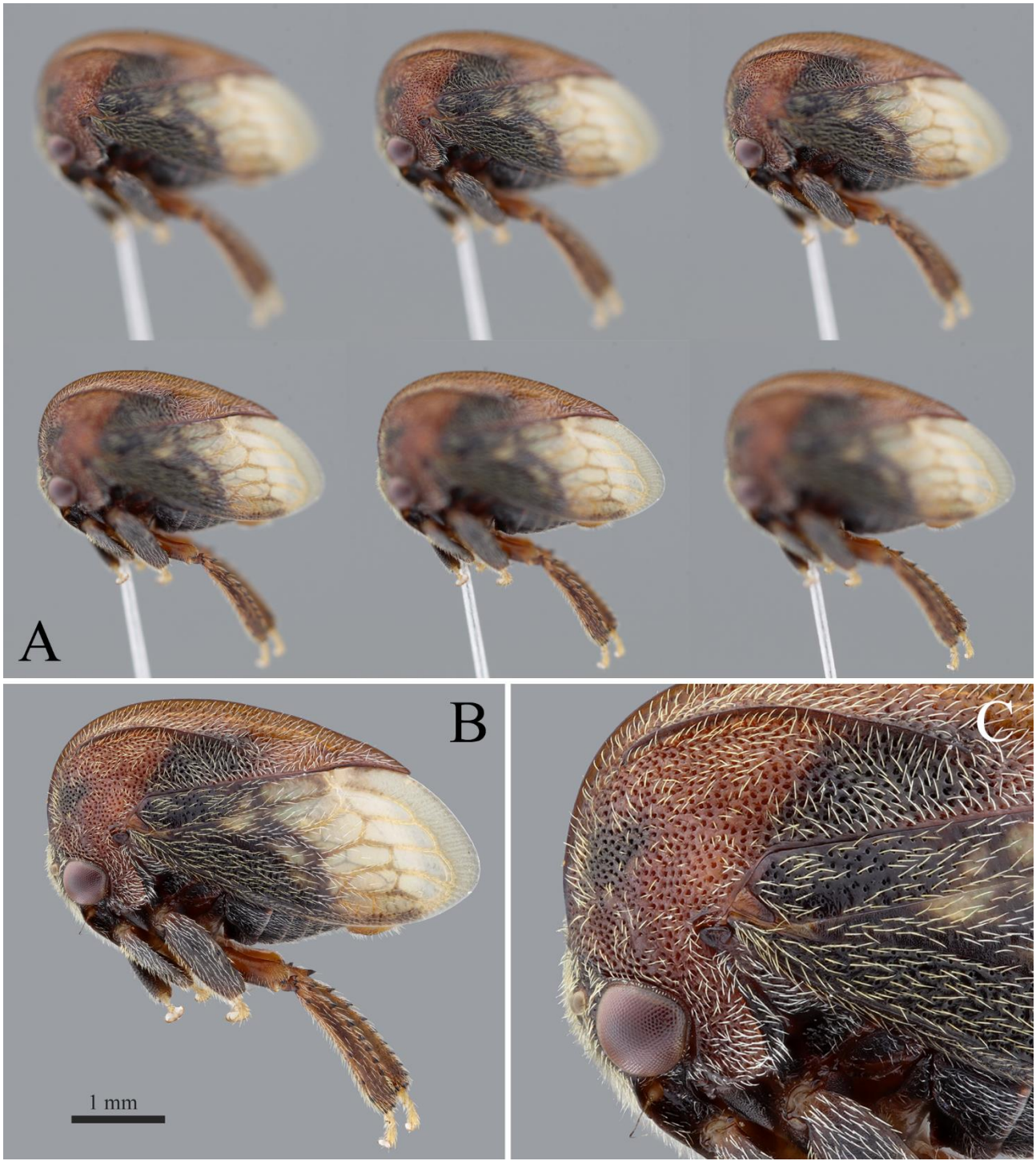

Figure 1. Demonstration of the image stacking process:** (A) Sequential images at different focal distances, (B) stacked image composed from the sequence of images, (C) Close-up of the stacked image. The number of digital images generated in the sequence will depend on the size of the specimen, ensuring that the structure of interest is completely covered without losing significant details of the cuticle and the specimen's body coverage. Photos: C. Flórez-V.

Materials y methods

The protocols described in this document condense the accumulated experience of the authors over years of seeking strategies to generate digital images across various entomological collections in Brazil (Museu de Zoologia da Universidade de São Paulo), Colombia (mainly at the Biological Collections of the Universidad CES [CBUCES] and the

Universidad del Magdalena [Unimagdalena]), the United States (The Frost Entomological Museum at Pennsylvania State University), and Puerto Rico (Invertebrate Collection at the University of Puerto Rico).

The example photos of insects presented here were taken using a focus stacking system. This technique involves capturing a sequence of multiple images of the specimen in the same position, altering the camera's focal distance (see the sequence summary in Figure 1). Subsequently, this series of images is stacked into a single image in which all focal planes achieve the highest clarity (Figures 1B, 2) using software programs.

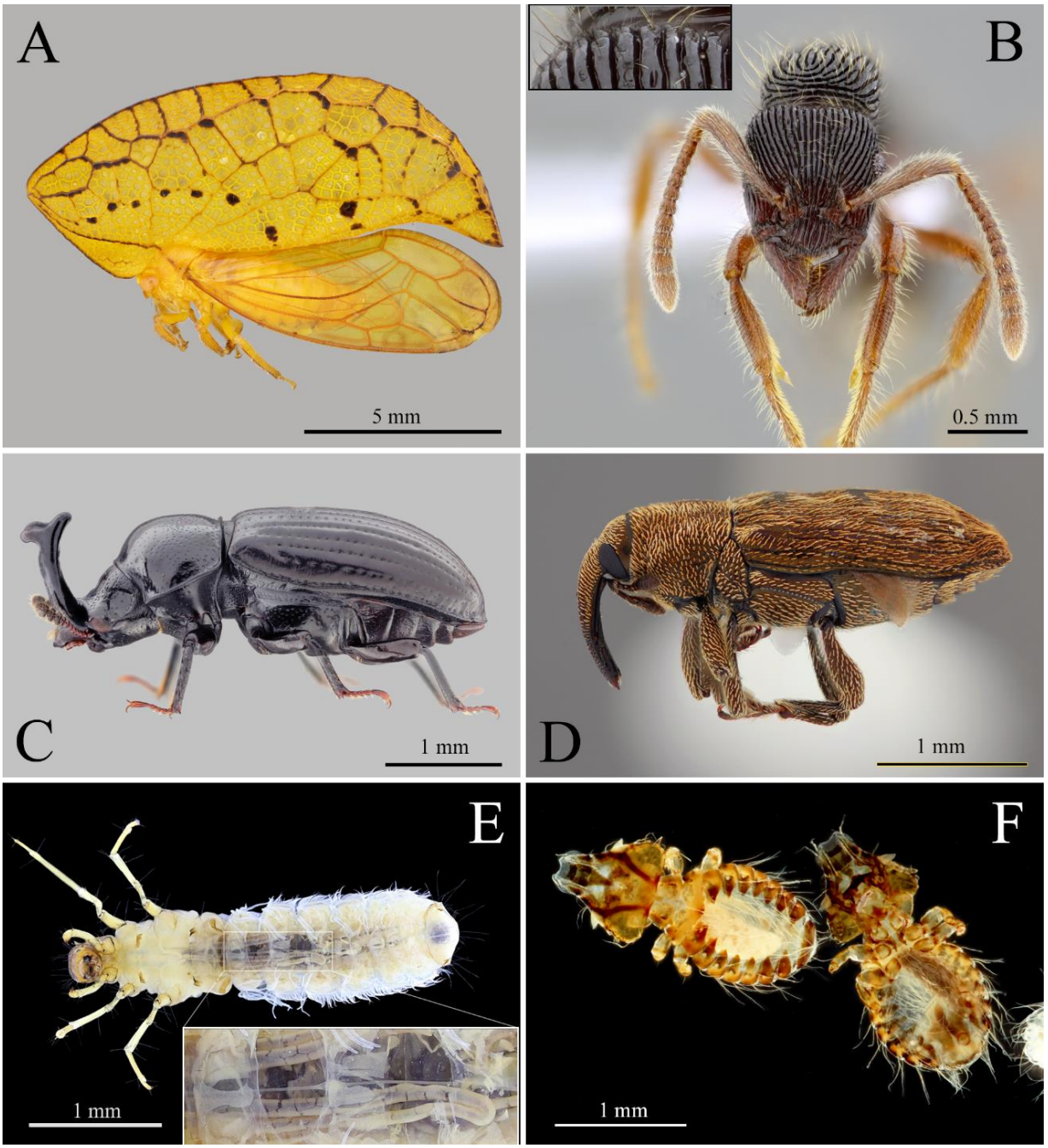

Figure 2. Insect photos were taken following the protocol described here. A) Oeda inflata (Hemiptera: Membracidae). B) Gnamptogenys strigata. (Hymenoptera: Formicidae). C) Tenebrionidae (Coleoptera). D) Baridinae (Coleoptera: Curculionidae). E) Phylloicus sp. (Trichoptera: Calamoceratidae). F) Phthiraptera (Psocodea). Photos E and F correspond to specimens photographed in liquid with a black background. Photos: C. Flórez-V.

The photographs were taken with a Canon MP-E 65 mm lens mounted on a Canon EOS Rebel T7i camera. Two methods were used to move these devices toward the specimen being photographed: the first used a base and a stereo microscope stand to hold and move the camera, manually adjusting the system at relatively equal intervals (Figure 3; enlargement in the Results section). The second method involved an automated mechanism, with a StackShot 3X Macro Rail (Cognysis: cognisys-inc.com) moving at pre-set distances, controlled from a computer via the Helicon Remote v. 4.3.1 program (Helicon Soft: www.heliconsoft.com) (Figure 4).

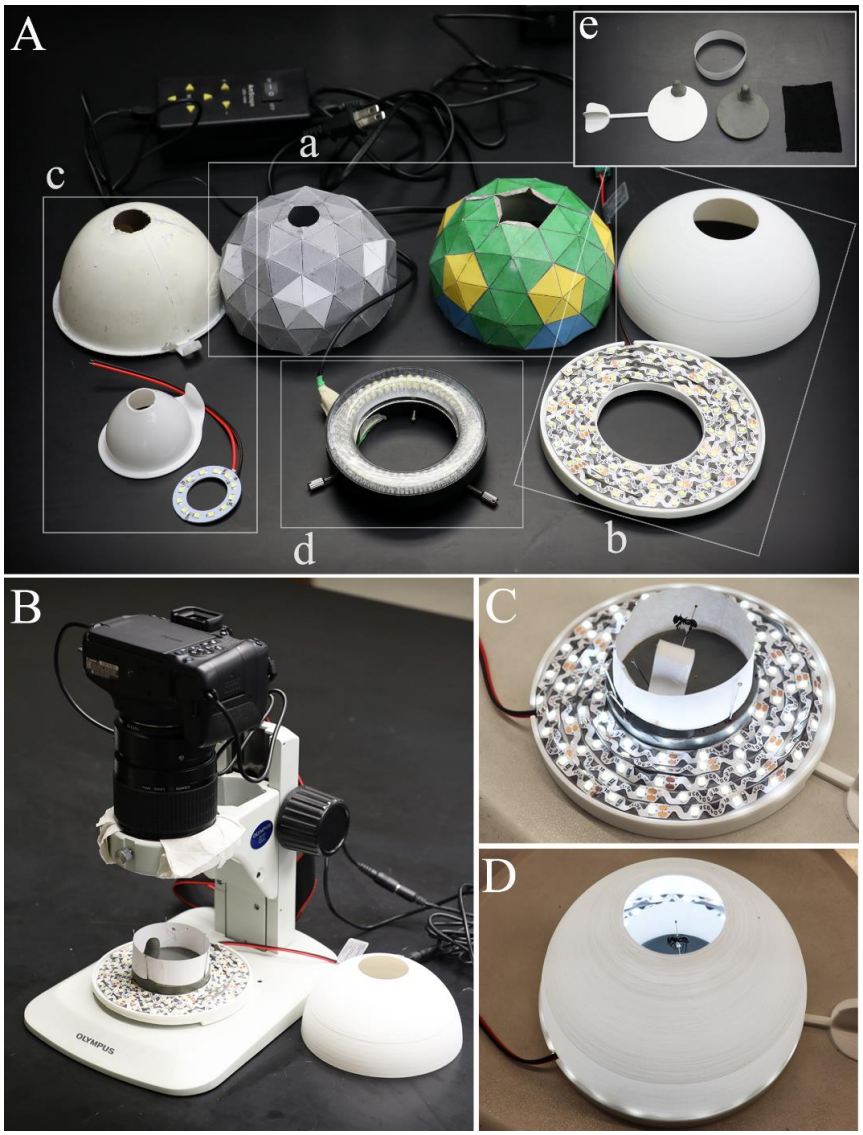

Figure 3. Lighting-related equipment: A) Domes, LED lights, and types of background used. (a) Domes designed following Kawada and Buffington's (2016) template; (b) 3D-printed dome and LED light built according to Bäulmer *et al.*'s (2020) indications; (c) Domes built from funnels and 40 mm diameter angel-eye LED light; d. AM Scope 144 LED Four-Zone ring; (e) background types (opaque white, standard gray, opaque black), moldable plasticine, and diffuser ring. B) A photography system based on camera and lens motion using a stereomicroscope support for taking multiple sequential images. C) The plasticine is used to position the specimen; the diffuser ring (indicated with the arrow) prevents the light from the LED ring from directly hitting the specimen. D) The dome reflects the light from the LED ring homogeneously. Photos: C. Flórez-V.

For lighting, an LED ring light (AM Scope 144 LED Four-Zone Microscope Ring Light) was used, covered with a paper dome constructed based on Kawada and Buffington's (2016) template. A system composed of SMD LED 2835 tape (Demasled: www.demasled.us) and a 3D-printed dome based on Bäumler *et al.*'s (2020) template was used. Moreover, a deflector ring was made from 90 g bond paper, parchment paper, or butter paper (Kawada and Buffington, 2016) to reduce glare and

allow for more uniform specimen lighting.

The computers used for all phases were an iMac with an M1 chip, 8 GB of memory, an eight-core CPU, and an eight-core GPU; a MacBook Air M1, 8 GB of memory, an eight-core CPU, and an eight-core GPU; and an Asus FX506LI-HN039 CI5 10300H laptop, with 16 GB of memory, a solid-state drive/90NR03T1-M04530, and a 4 GB GTX 1650 Ti video card.

The images were stacked using Helicon Focus v. 8.2.3 (Helicon Soft: www.heliconsoft.com) and subsequently edited in Adobe Photoshop®. The notation used for the editing process to specify tools, uses, and commands in Photoshop for Windows and MacOS will be detailed in the Results section. Advanced tool functions can be consulted in the Photoshop user guide.

Results

The following presents a general protocol and recommendations for performing focus stacking using lighting from an LED ring diffused by a geodesic dome (Bäumler *et al.*, 2020; Kawada and Buffington, 2016) and two camera movement systems, either through motorized rails (automated) or manually, with the help of a stereo microscope stand.

Several considerations are proposed regarding the selection and preparation of specimens, lighting, image backgrounds, camera setup, and the handling of various software and stacking systems to ensure good quality and usefulness of the image sequence generated with different focal distances (Figure 1A) used in the final composition (Figures 1C, 2). General recommendations are also provided for the subsequent processing of the images (e.g., editing and storage), as well as the infrastructure, equipment, and software costs. Additionally, considerations are made regarding the necessary personnel time. Table 1 can be used as a reference to navigate the document.

Table 1. Contents of the presented protocol, including general preparation aspects, the procedure for photographing specimens mounted on entomological pins, and other important considerations for applying the protocol included in this article.

1. General aspects of preparation for the protocol
1.1. Lightning
1.2. Camera Settings
1.3. Focus stacking systems
1.3.1. Manual
1.3.2. Automated with the use of motorized rails
1.3.3. Other considerations for stacking system selection
1.4. Programs to control the camera and stack images
2. Photographing protocol for entomological pin-mounted specimens
2.1. Equipment preparation, specimen preparation and cleaning, and connection verification
2.2. Configuring the camera from the computer to generate images at different focal lengths
2.3. Generation of the image sequence at different focal lengths
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1. General aspects of preparation for the protocol

1.1 Lightning

For arthropod photography, it is essential that the light does not come directly from the source but is somewhat diffused or comes indirectly. For this purpose, LED rings concentrate and diffuse the light through a geodesic dome (Kawada and Buffington, 2016). These two devices are economical compared to flash lighting systems, offering equal or superior results.

Indeed, Kawada and Buffington (2016) established a scalable and modular lighting system that allows for the construction of domes and LED rings of various sizes according to needs (Figure 3A). Other low-cost options include domes made from 3D printing and moldable LED lights, such as those proposed by Bäumler *et al.* (2020) (Figure 3A, b).

The mentioned systems allow for the creation of domes and LED rings that concentrate light more effectively depending on the size of the specimens. The smaller the insect, the more light will be needed because higher magnification reduces the amount of light reaching the camera sensor. Additionally, with higher magnification, the focal distance must be shorter (for the Canon MP-E 65 mm lens), which means the lens must be positioned closer to the specimen.

The above becomes much more critical when using very high magnifications, as the focal distances are very short and require smaller domes and rings. Fortunately, Kawada and Buffington (2016) and Bäumler *et al.* (2020) provide solutions for small sizes of these elements, which can be used with high magnifications such as those provided by microscope objectives (Figure 3A).

If the lighting is homogeneous and diffuse, some areas of the cuticle's sculpture may appear flattened, and certain reliefs may not be distinguishable, which can be due to the lack of direct light, a disadvantage of this system. However, this can be partially addressed by using domes with some portions painted opaque black (Kerr *et al.*, 2008), which allows for an uneven distribution of light inside the dome and creates some shadows.

The paper deflector ring is crucial for preventing direct light from the LED ring from reaching the specimen, maintaining texture and setae edges, and preventing the loss of details, especially in very smooth and shiny specimens. This device can be made with white bond paper of various weights, although those that specifically prevent direct light while allowing for diffusion (between 75-90 g) are recommended (Figure 3A, e).

Systems that use flashes also allow for good illumination of specimens if a light diffusion device accompanies them. In these cases, using camera lights in conjunction can be favorable. One of the advantages of this alternative compared to domes is that the light is directed, which allows reliefs and sculptures to be seen in the integument. They can also be more beneficial in photographing insects in liquid since they reduce the manipulation of specimens and movement around them. However, flashes generally cost more, must be left to rest between photo sequences, require replacement over time due to their useful life, and demand investment in batteries and charging systems.

1.2 Camera Settings

This section explains some fundamental aspects of photography, including how to configure the camera to obtain high-quality images with correct lighting and color.

ISO, aperture, and exposure time are the elements of the camera that allow managing light in photography. ISO is the sensitivity of the camera sensor to light. Thus, the higher the ISO number, the greater the sensitivity and better the ability to capture light. However, this translates into image quality loss (generating noise and a grainy effect). Therefore, even though the sensor is less sensitive to capturing light at lower ISOs, the image has less noise and higher quality.

Opening the diaphragm allows you to increase or decrease the depth of field (i.e., the focused portion of the image). In the first case, you must close the diaphragm (higher f-numbers), letting less light enter the camera sensor. In the second case, you open the diaphragm (lower f-values) so that the camera captures more light.

Exposure time refers to the span the shutter is open, allowing light to reach the sensor and capture the image. Therefore, as this period increases, the camera receives more light. However, if the camera or the specimen moves, the image will be blurry and undefined. On the contrary, the camera will capture less light if the shutter is open for less time. However, the image will

be clear if the camera or specimen movements are imperceptible.

With these aspects of light management, a balance can be achieved to obtain good image quality. In collection photography, the objects are stationary, and the camera is attached to a static support (see below for recommendations to reduce vibrations in the system); therefore, photos can be taken with relatively high exposure times (up to 1/15), ideally keeping the ISO between 100 and 200, for a good-quality image.

However, it should be noted that as the magnification increases, the light received by the camera decreases, and the effect of vibration or movement in the photo becomes more evident. Thus, the need to reduce the exposure time can be compensated by opening the diaphragm (as little as possible). The depth of field will be reduced, and more photos will have to be taken at a shorter distance to capture the detail of the surface.

White balance is also critical to maintaining the actual or natural colors of the specimens (Allen and Triantaphillidou, 2012). This balance will depend significantly on the lighting source and its color temperature (e.g., warm (yellow) or cold (blue) light). Cameras have options to perform this balance automatically, and in some cameras, it can be done manually, or you can choose between several options to neutralize the color temperature. It is recommended to have lighting sources with a neutral color temperature between 5,200-6,000 k (these specifications are found in most lighting sources). This way, the images will preserve their natural colors, and time will be saved during the editing process after producing the final image. Another option is to create color profiles using color cards, which allows you to configure and obtain a precise color given a standardized form of lighting.

1.3. Focus stacking systems

Depending on the lens and camera, there are different ways to perform focus stacking. For lenses with fixed or manual focus distance (depending on magnification), such as the Canon MP-E 65 mm, the Laowa 60 mm, or the Laowa 25 mm, the camera or object must be moved in and out to take photos focusing on different areas of the specimen. This back-and-forth movement can be done manually (using a stereomicroscope stand) or automatically (using a motorized rail).

1.3.1. Manual

In this method, the camera is attached to a stereoscope support and moved using the focusing screw. In this case, a stereoscope with a head detachable from the base is required (Figures 3B, 3C). As with the automated system, it is necessary that the stereoscope support is firm and the camera is well attached to it.

Focusing screws can be loosened or tightened, depending on user preference, to make movement between each photo smoother. Usually, this is achieved by turning the two sides of the screw in opposite directions, in which case it is recommended to check the characteristics stereomicroscope's base. Paper (figures 3B, 3C) or even the lens hood can be used to fit the camera to the support better.

An advantage of this system is that, with some skill, smooth movements between each photo can be made without generating vibration, which is convenient for recording small specimens (less than 8 mm) that require higher magnification (4X-5X) and more light or longer exposure times.

On the other hand, one of this method's disadvantages is that it requires skill and experience to achieve precise (i.e., the same distance used in each step) and short adjustments of the focus screw. Also, the turning of the focus screw must be much finer as the magnification is increased. Regardless, achieving the necessary skill for this process is possible after a few hours of practice. However, unlike the motorized rail, there is a tendency to overestimate the number of photos taken (more steps than necessary, which leads to a more significant investment of time and space used on hard drives).

1.3.2. Automated with motorized rails

This technique uses motor-driven rails programmed to make movements of identical length (steps) from the insect's parts closest to the lens to those furthest away. Each photo has an area that overlaps with the previous one so that the software can stack the photos. The most commonly used rails are Cognysis StackShot and Wemacro Rail (www.wemacro.com), which are usually installed on a firm support (e.g., stand copy), where they remain in a vertical position, while the specimen is placed on the base of the support (figures 4A, 4E).

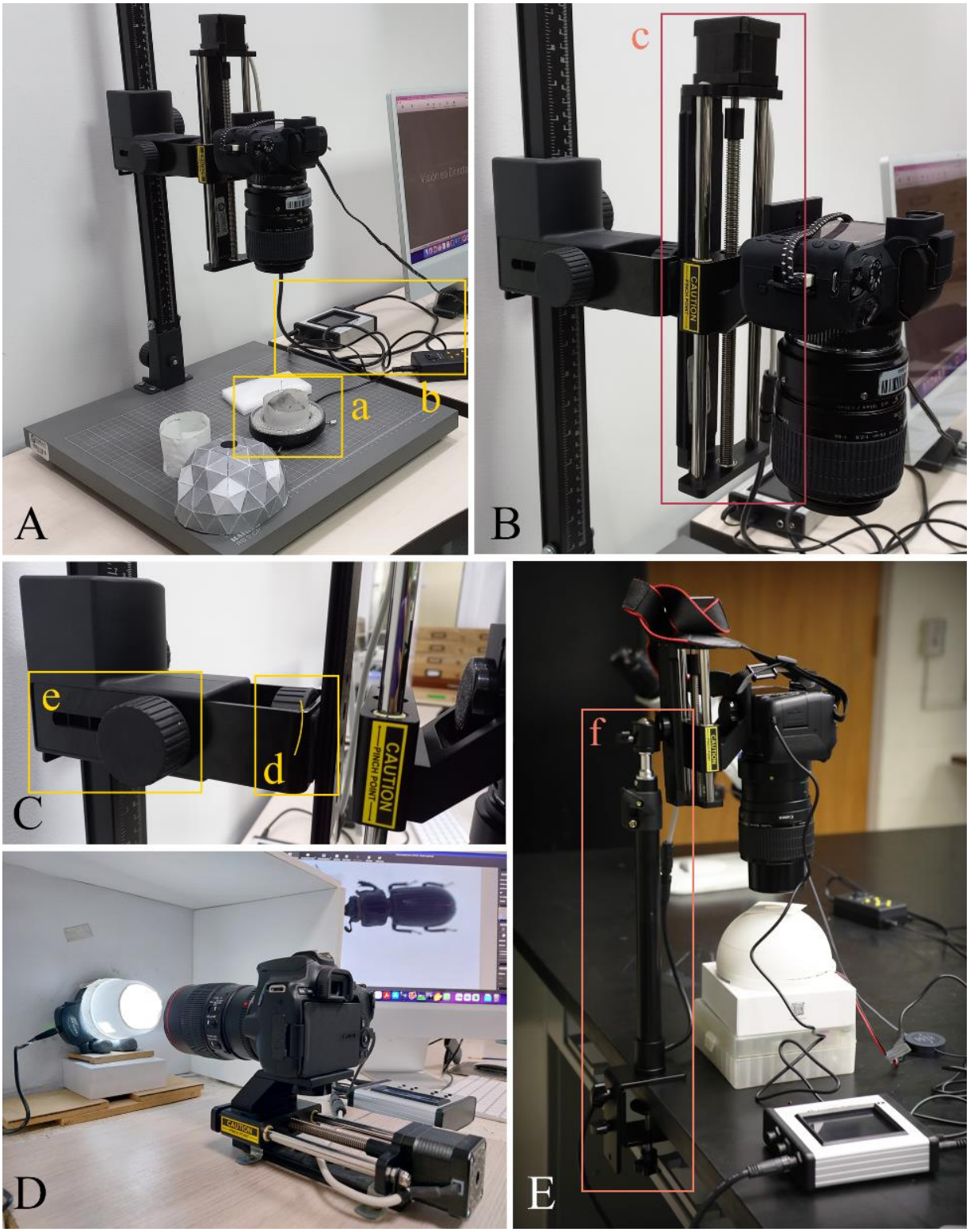

Figure 4. Automated camera and lens movement system using a motorized rail to generate multiple sequential images: A) Support for the system for vertical positioning. (a) LED ring light; (b) Arrangement of cables and system accessories. B) Detail of the coupling connecting the rail (Macro Rail) to the support. (c) Rail in a horizontal arrangement. C) Detail of the support at the rail anchor point. (d) Screw securing the support to the rail; (e) Retracted position of the support arm to reduce torque. D) Horizontal arrangement of the system. E) Alternative for the vertical arrangement of the system, with (f) a table press support with a screw. Photos: J. Cardona-Duque (A-C), L. Jiménez-Ferbans (D) and C. Flórez-V (E).

Alternatively, the rail can be supported by a horizontal support, placing the specimen vertically in front of the lens (Figure 4D). Using the illumination system with the dome in this last installation is more complex, but other options (e.g., flashes and softboxes) are also possible, and there are more possibilities for installing this system where the camera remains stationary while the specimen moves using the rail.

An advantage of the automated system is that it optimizes the number of steps between each photo and allows the possibility of adjusting the movement distance between records, which can make taking photos more efficient and does not require more skill than the manual system. It is also advantageous with large specimens (larger than 8 mm) as they require slight magnification and less lighting, and the camera can be set to shorter exposure times.

This method's disadvantage is that it can generate vibrations with the motor's movement, so it is recommended to use a stable and robust support and increase the pause time between photographs as the magnification increases. The rail settings can also be modified to adjust the force and speed with which it moves to smooth the movement and reduce vibrations (see the StackShot manual [cognisys-inc.com] for Tramp, Torque, and Hi-Precision properties).

This automated alternative is also relatively portable, depending on the medium, so it can be transported (Figure 4E) to other collections and even to field sites with access to electricity, giving researchers freedom when they travel to review material at other museums. However, it is more expensive than a manual system.

It is necessary to consult the lens's depth of field at each magnification to calculate the length and number of stops (Brecko and Mathys, 2020). This information can be found in each of the lenses manuals. In principle, it is ideal to maintain the effective aperture (f-). However, since the type of photography discussed here requires more light input to maintain an ISO of 100-200, the aperture should be between 2.8 and 5.6, depending on the intensity of the lighting system. According to the manual for the Canon MP-E 65 mm, for example, the depth of field at 1X magnification and 2.8 aperture is 396 µm. Thus, smaller steps should be chosen to ensure there is some overlap between each of them. In this case, steps of 300-350 µm would be selected. Also, the depth of field at 4X magnification and a 2.8 aperture is 62 µm, so a step distance of 40-50 µm should be used.

The step distance can be set in Preferences > Shooting > StackShot under "Focus step size." After selecting the area closest to the lens (A) and the furthest (B), Helicon Focus will automatically calculate the number of shots between these two points. Brecko and Mathys (2020) indicate that the average number of steps to generate a final stacked image is between 15 and 40 if these recommendations are followed.

Another alternative method is to use the autofocus feature of some cameras and lenses without moving the entire system (Mertens *et al.*, 2017). This can be achieved, for example, with the Canon 100 mm Macro IS USM and using the Helicon Remote to choose the closest and farthest location of the specimen and configure the number of steps necessary. However, this article will not expand on this technique since the authors have not tested it. It should be noted that using this method with the fixed focus distance lenses mentioned above is impossible since it must be focused manually. Mertens *et al.* (2017) discuss using this system in detail, even employing compact cameras of much lower prices.

1.4 Applications for controlling the camera and stacking images

There are several programs for adjusting camera settings from your computer (Figure 5) and executing focus stacking (Figure 6). A free option for Windows is Combine ZP, which allows you to align and stack images using several different methods. It should also be noted that this option only stacks images and does not allow the camera to be controlled from the computer, so you must use a separate application for the camera (e.g., Canon EOS Utility). Also, Combine ZP does not work well with huge files (e.g., RAW photos) or large photo batches.

You can change camera settings (e.g., shutter speed, ISO, aperture) and take previews and photos from the Canon EOS Utility. However, you cannot program it to take photos automatically without pushing the shutter button for each shot. Using the StackShot rail, you can set up the camera from the Canon EOS Utility and program

shots directly from the rail.

On the other hand, some applications allow controlling and configuring the camera from the computer (exposure time, ISO, and aperture) and image stacking. Acquiring software licenses represents a cost, but these might be perpetual with the right to updates and sometimes allow installing the program on multiple computers. Helicon Focus and Zerene Stacker (www.zerenesystems.com) are the most common of this type, with which you can control and configure the camera and the StackShot rail and import and stack photos.

Helicon Focus is faster than Zerene Stacker at focus stacking; however, Zerene Stacker allows you to align the photos before stacking, which can be very convenient when taking pictures of specimens in liquid, where there is slight movement (Brecko and Mathys, 2020). Both programs also allow editing the image after stacking photo groups (Figure 6C). Each has different stacking methods for different purposes; in particular, the C (Pyramid) Method in Helicon Focus and the PMax in Zerene Stacker are the best for capturing specimen images (Brecko *et al.*, 2014). Despite their cost, these two options make visually recording arthropods in collections much easier and produce a much cleaner and sharper stack than Combine ZP.

Finally, these programs can be configured to do multiple batch stacking (Figure 6B) to get sequences of photos from different specimens or views and stack them later. This method allows the computer to run the stacking at the end of the day and thus optimize the time spent taking pictures, which is convenient if you are entirely sure that the image captures are being done correctly.

2. Photographing protocol for specimens mounted on entomological pins

The following equipment and materials are required to follow the protocol below:

1) Canon Rebel T7i camera (or any Canon interchangeable lens camera with APS-C sensor).
2) Canon MP-E 65 mm lenses (for specimens between 2-25 mm) or Canon EF 100 mm f/2.8L Macro IS USM (for specimens larger than 25 mm, in manual focus).
3) AM Scope 144 LED Four-Zone Microscope Ring Light (or any illumination system using LED lights or rings; see illumination section and figure 3A).
4) StackShot 3X Macro Rail (Cognysis) motorized rail or stereo microscope stand (a low-cost alternative to the rail, Figure 3).
5) 2020 MacBook Air M1 computer (or any computer with sufficient processing power; see Helicon Focus specifications at www.heliconsoft.com).
6) Standard-sized geodesic dome, designed as proposed by Kawada and Buffington (2016) (Figure 3 A, a) or Bäumler *et al.* (2020) (Figure 3 A, b), although an alternative may be a hemispherical funnel with a white interior (Figure 3 A, c).
7) Two USB A to Mini B cables.
8) Standard grey clay (closest to the 18 % grey found on photo cards; Figure 3 A, e) and a plate (can be a small Petri dish) to hold the clay and mold it as needed.
9) Deflector ring (made of 90 g bond paper, parchment paper, or butter paper; figure A, e).
10) For the system using motorized rail, photography support (figure 4a) or base for a horizontal arrangement of the rail (figure 4d).

The steps for each activity in the protocol are described sequentially in the following subsections.

2.1 Equipment preparation, specimen preparation and cleaning, and connection verification

1) Clean the equipment and make sure the camera lens and sensor are clean.
2) Ensure the camera battery is charged (some adapters allow the camera to be connected to alternating current).
3) Install the LED ring, ensuring the cables do not interfere with other equipment (figure 4a).
4) Place the plate with grey molding clay (checking that the base is clean and the surface is homogeneous, particularly in the area where the photo's background will be) and place the deflector ring near the lighting source.
5) Prepare and clean the specimens, as described later in this methodology.
6) To connect the equipment, it is recommended that you follow the steps described in Table 2, depending on the support you will use to move the camera.

Table 2. Steps for connecting equipment according to the type of support available in the collection for moving the camera while generating sequential images.

If a stereomicroscope stand is used	If a StackShot rail is used
i) Attach the camera to the stereoscope support, ensuring it is firm to avoid vibrations (figure 3B–C).	i) Attach the rail to the support (if a support is used, it is recommended that the arm be retracted to reduce torque; Figure 4C) and it is secured firmly (Figure 4C); it is also recommended to check with a bubble level if the support is completely vertical (Figure 4B).
ii) Connect the camera cable to the computer (USB A to Mini B cable).	ii) Attach the camera to the rail and secure it firmly.
iii) Place the LED ring and the plate with grey molding clay under the camera.	iii) Connect the rail controller and camera cables to the computer (USB A to Mini B cables). Connect the rail cable to the rail controller. Connect the rail adapter cable to the power outlet.
	iv) Turn on the rail (before opening Helicon Remote, which allows the rail connection to the computer to be recognized).
	v) Place the LED ring with the plate with grey molding clay in front of the camera.

2.2. Configuring the camera from the computer to generate images at different focal lengths

1) Open Helicon Remote on your computer (figure 5).
2) Turn on the camera and wait for the program to detect it. If this does not happen, you can manually search for it in the "Camera settings" menu in the upper right corner of the program window, which allows you to change the camera settings (e.g., ISO, aperture, exposure time) from the computer (it will not be possible to modify these parameters from the camera).
3) Activate live view.
4) Set the camera to automatic ISO so you can observe the specimen while adjusting the position (this ISO can be modified later).
5) Locate the insect and place it in the desired position using grey molding clay. It should be as central as possible (tracing the circumference of the LED rings). The specimen can be focused using the rail by moving the camera closer or further away, employing the arrows in the "Focus Bracket" section of the program. If the stereomicroscope stand is used, this camera can be moved closer or further away by adjusting the focus screw.
6) Adjust lens magnification. The specimen should be magnified as much as possible, leaving some free space at the edges.
7) Set the image quality to fine (the highest detail in JPEG) or RAW according to preference (note that while RAW may yield better quality, the storage space requirement may be enormous).
8) Install the deflector ring, ensuring no direct light passes through the LED rings (Figure 3C).
9) Place the dome to cover the LED ring, the molding clay, and the specimen while avoiding touching the latter (figure 3D).
10) Turn on the LED rings.
11) Change the ISO to 100 and set the shutter speed and aperture. To avoid camera shake and blurry photos, you can leave the aperture as wide open as possible, i.e., low f-stops, and exposure times of more than 1/15 if you use the manual system or more than 1/25 if you use the rail (1/40 for tiny specimens, less than 8 mm). Since increasing the aperture decreases the focal length, taking a larger number of images is recommended, i.e., reducing the number of steps between shots (see stacking section). Also, if there is insufficient light to maintain these exposure times, it is preferable to use an ISO of 200 while keeping the shutter speed high.
12) Set the white balance to obtain a neutral color temperature (figure 5 A, k).

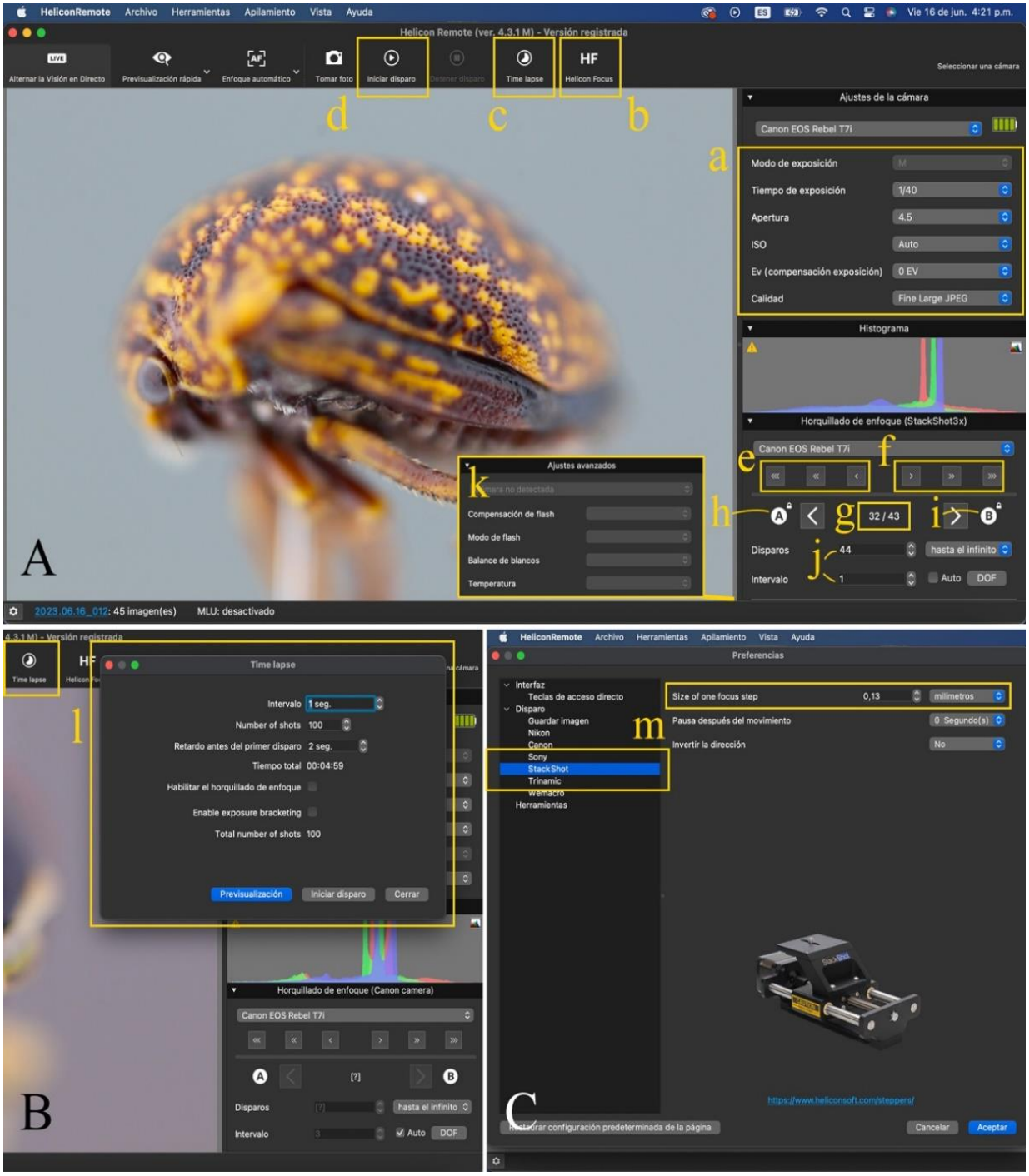

Figure 5. Helicon Remote workspace. A) Default workspace, B) 'Time Lapse' options panel, C) StackShot rail settings panel (Helicon Remote > Preferences > StackShot). (a) Exposure time, Aperture, ISO, and Quality settings. (b) 'Time Lapse' option to open panel 'l.' (c) Option to open Helicon Focus to stack the sequence of photos; d. The "Start Shooting" option allows the program to automatically take photos and move the rail according to the 'Focus Bracketing' settings. (e and f) Rail shift controls to move the camera away from or closer to the specimen, respectively. (g) The program's number of steps is based on the step size setting in 'm' and the distance between the closest (h) and farthest (i) points. (j) the number of shots or frames the program will take and the distance interval between each photo; it is recommended that the interval be 1 and that the number of shots is equal to or slightly greater than 'g.' (k) The "Advanced Settings" panel is used to configure the White Balance and Temperature. (l) The "Time Lapse" panel allows configuring the number of shots and the time interval when using the stereomicroscope support. Photos: C. Flórez-V.

2.3. Generation of image sequences at different focal lengths

2.3.1. Using the stereomicroscope stand

(Figure 3, 5; watch video in annex 1)

1)Move the focusing screw on the stereomicroscope stand to view the parts of the specimen that are closest and those that are farthest from the camera focal plane.
2) Raise the camera until the specimen is entirely out of focus and lower it slowly until the part closest to its focal plane is focused.
3) Click on "Time Lapse" (Figure 5A, b). In principle, you could select between 70-90 photos, which is an overestimate since it is impossible to know how many shots are needed. Also, set a time between one-second shots (if you lack skill, it is suggested to set this option to 2 or 3 seconds) and another for the first recording of 2 or 3 seconds so you have time to manipulate the stereomicroscope's focusing screw (Figure 5 A, k).
4) Click "Start," and the camera automatically takes the pictures. After each shot, gently (and in as short a step as possible) turn the focusing screw (the higher the magnification, the smoother the movement should be). After taking the picture from the specimen's farthest point, click stop and close.
5) Click on Helicon Focus (figure 5A, c).

2.3.2. StackShot Rail

(Watch the video in annex 2)

1) The distance of each step is set depending on the lens' magnification (see section 1.3.2., Figure 5C, m)
2) Determine the part of the specimen that is closest to lens "A" (higher if the holder is vertical) and the part furthest from lens "B" (lower if the holder is vertical). It is recommended to raise the rail until the specimen is out of focus, lower it slowly until the upper part is in focus, and then click "A" (Figure 5A, h). Likewise, for the lower part, set it by clicking "B" (Figure 5A, i). Helicon Remote automatically sets the number of steps, given the configured focus step distance (see Stacking section, Figure 5A, g).
3) Click "Start shooting" (figure 5A, d). The track will go to A (the zone configured as the closest) and start taking pictures automatically, moving each time you shoot. The program will take the last photo at B (the zone configured as the farthest).
4) Click on Helicon Focus (figure 5A, c).

2.4. Stacking photo sequences

These steps apply equally to the two previous systems (Figure 6).

1) With the Helicon Focus program open, you must stack the photos from the sequence generated in the previous steps. These captures are usually loaded when you click "Helicon Focus" after taking the pictures in Helicon Remote. However, if these do not load automatically, you must open the folder where the photos were saved, select them, and drag them from the folder to Helicon Focus or use the "Add images" menu (figure 6).
2) Make sure that all photos are selected in the application.
3) Select "Method C (Pyramid)" (check that smoothing is set to level 2) (for details about stacking methods, see Brecko *et al.*, 2014).
4) Click "Render." The program will show how the focused areas are stacked in each photograph.
5) Once the stacking process is complete, click on the image to view a close-up. Check that the various structures are in focus to see if retaking the sequence of photos is necessary.
6) If areas are overlapping due to stacking (Figure 6C), it is possible to edit the photo in the "Retouch" tab, where you select the image you want to remain in focus and configure the brush size, color tolerance, and hardness.
7) Save the image in TIFF format by selecting the folder and naming the file (see the next section for recommendations for naming the final stacked image file).
8) Pay attention to the magnification at which the photos are taken if using the Canon MP-E 65 mm lens to generate the scale later (see section on generating the image scale below). Photograph only one ruler without moving the focus when using Canon EF 100 mm lens.

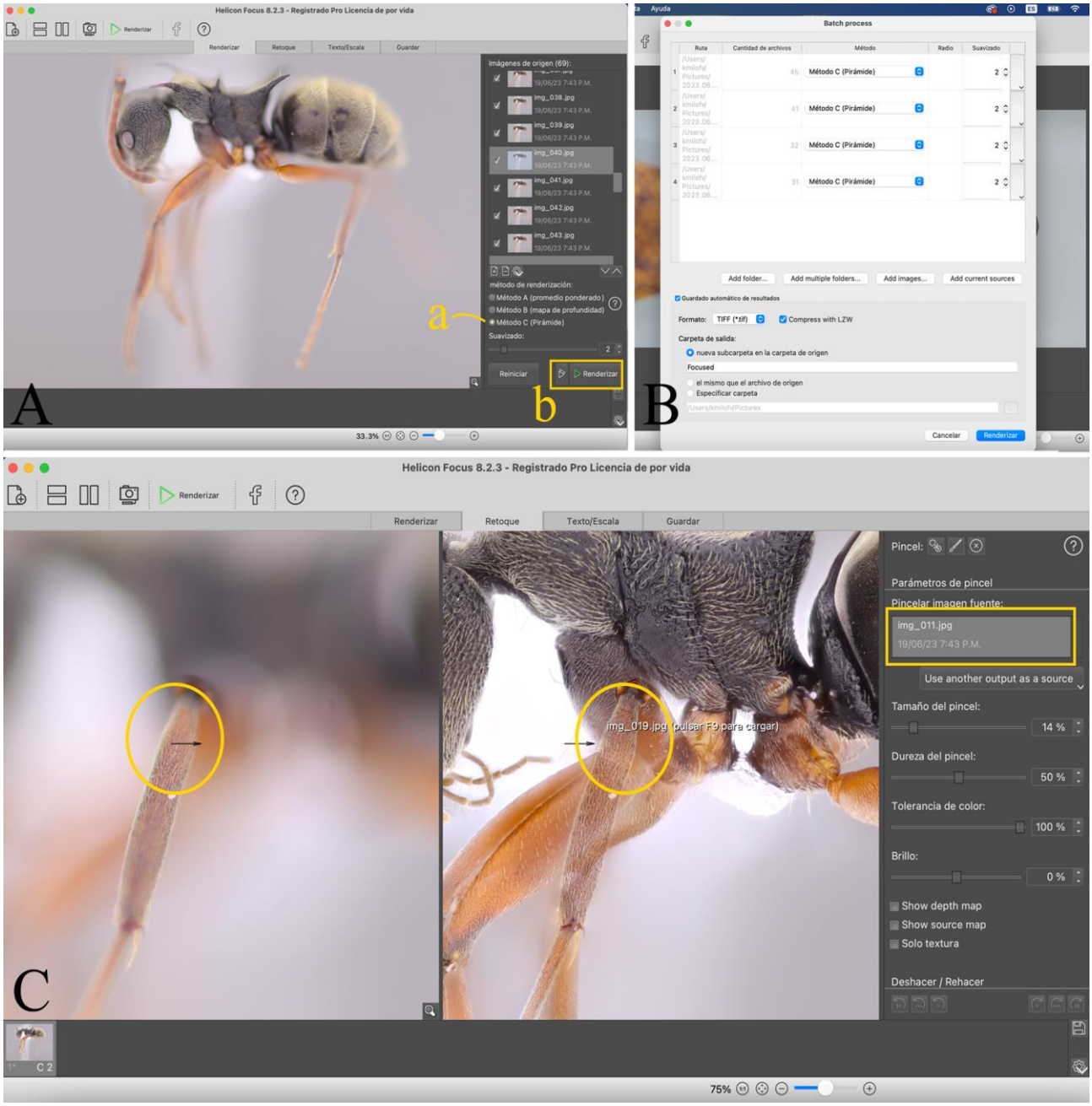

Figure 6. Helicon Focus workspace. (a) Default workspace; (b) Recommended stacking method ('Pyramid'); (c) Starts the image stacking process; (d) Patch stacking process panel (File > Batch Process); (e) "Retouch" editing workspace option, for choosing the focused areas in overlapped images in the stacking process. Photos: C. Flórez-V.

2.5. Recommendations when naming the final image file

Once the stacked image has been obtained, the file must be saved. It is generally recommended that photographed specimens have a catalog number in the depository collection. This number must be included in the output file name with its corresponding collection acronym. In addition, it is suggested that names not contain spaces, punctuation marks, or special characters other than the hyphen or underscore (Figure 7).

On the other hand, if there is a previous or definitive identification of the specimen, it should be included in the file name. Likewise, it is advisable to add information such as specimen orientation in the image (e.g., lateral, frontal) or the specific morphological structure (e.g., right posterior tibia),

the lens magnification used (e.g., 1X, 4X) and the initials of the person who generated the image (e.g., CFV). This last piece of information, in particular, is suggested because, generally, in countries such as Colombia, intellectual property regulations consider moral rights over a work inalienable, imprescriptible, and unseizable (article 30 of Law 23 of 1982). For this reason, the author of a work of human intellect, such as photography, always keeps the right to have his name mentioned when his production is being used.

When using initials in the collection, it is advisable to have a file (at least in Excel) linking letters to the full names of the work's creators. This way, assigned file names can be kept with variations in subsequent modifications. It is also recommended that collections store a file where the specimens that have been photographed can be tracked.

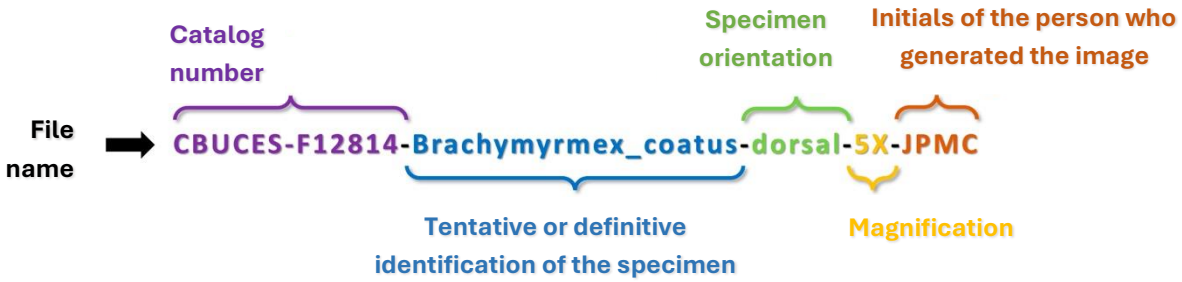

Figure 7. Recommended nomenclature for naming image files Prepared by: J. Cardona-Duque.

2.6. Image scale generation

Indicate the insect's scale in the stacked image to provide information on the specimen's size and proportion, allowing comparisons with other specimens or structures. For this, Brecko and Mathys (2020) suggest two methods using a lens with a fixed focus distance.

The first option is to photograph a ruler at different magnifications, giving the equivalent number of pixels of a given measurement at each magnification (Figure 8). The other method is to calculate the number of pixels that correspond to 1 mm using the equation (m/s)*w, where m= the magnification, s= the sensor size (mm), and w= the maximum width of the image in pixels (this feature can be found in each camera's specifications as the "maximum image resolution"). Thus, a photo editing program can create a bar of the desired pixel numbers according to the required size (Figure 8).

3. Other aspects to take into account during protocol application

3.1. Selection, cleaning, and preparation of specimens

It is vital to select whole specimens that are intact and properly mounted. Recently collected insects must be mounted to allow observation of their diagnostic characteristics in the photograph. This mounting can be similar to the general indications for each study group, such as that of Martin (1977).

Likewise, cleaning the specimens to be photographed allows for better observation of specific characteristics (e.g., setae, scales, sutures) and considerably reduces the time spent editing photos. Fine brushes previously moistened with distilled water or 70 % ethanol help remove dust from dry specimens, after which specimens must be dried completely.

However, cleaning will depend on the arthropod's fragility or the area treated since many of these organisms' legs and antennae can be exceedingly brittle, even in contact with fine brushes. In these cases, it is preferable not to clean the specimen and invest more time in the photograph's final editing. This option is crucial for insects with few individuals or specimens of high taxonomic value (e.g., types).

For specimens preserved in alcohol, cleaning can be done by passing a soft brush over the specimen and renewing the liquid. Some diagnostic structures of these insects can be very fragile (e.g., gills of Ephemeroptera), even more so than those of dry arthropods, so it is advisable not to clean them in these cases.

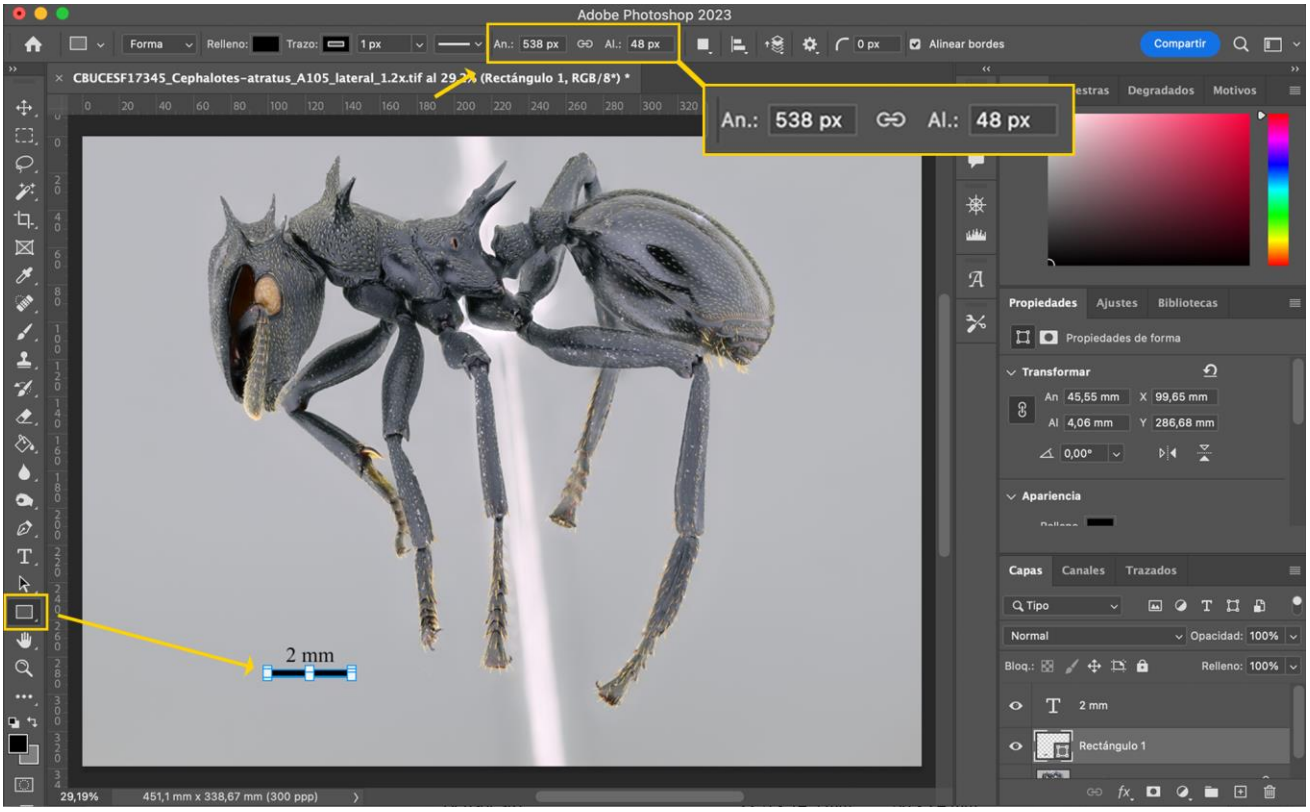

Figure 8. Generating the specimen scale in Adobe Photoshop. (A) Photo at 1x magnification (m), taken with a Canon T7i camera with a sensor size of 22.3 mm (s) and a maximum image width of 6000 pixels (w). Thus, using the formula to obtain the number of pixels corresponding to 1 mm = (m/s)*w, we obtain that 269 pixels correspond to 1 mm. To set a scale of 2 mm, we multiply by two and select the number of pixels we want for the width in the 'Rectangle' tool, in this case, 538 pixels. It is recommended to use an equal number for the height of the photos (at least in the same views) so that the scale remains homogeneous. In this case, 48 pixels were selected arbitrarily. Photo: C. Flórez-V.

It is also suggested that the entire setup of the photography system be cleaned before using it to prevent dust or other particles from adhering to specimens, which is even more relevant when recording insects in ethanol (see section on specimens in liquid). Likewise, it is vital to keep the lenses clean since any dirt on them will be magnified in the image, may cover some essential details, and increase editing time.

3.2. Positioning of specimens

Devoting time and care to position the specimen is essential, as it is impossible to rotate it once the stacked image is generated. For digitization of pin-mounted insects, it is recommended to have at least complete photographs of specimens in frontal, lateral, and dorsal views (even in some scenarios, it is mandatory to have all three panoramas contribute to image banks; e.g., AntWeb: https://www.antweb.org/).

For the lateral view of pin-mounted specimens, it is convenient to take photos from the left side to avoid obstructions. However, the selection of specimens and their orientation in the images will depend on the purpose for which they are generated. For example, images intended for a scientific publication may include different views and details of specific morphological characters requiring greater magnification.

Tiny but conspicuous errors are common when positioning the specimen (Figure 9A), so you must ensure the insect is in the required position before taking the photographs; for example, completely dorsal, lateral, or exposing exactly the morphological structure to be shown (Figure 9B).

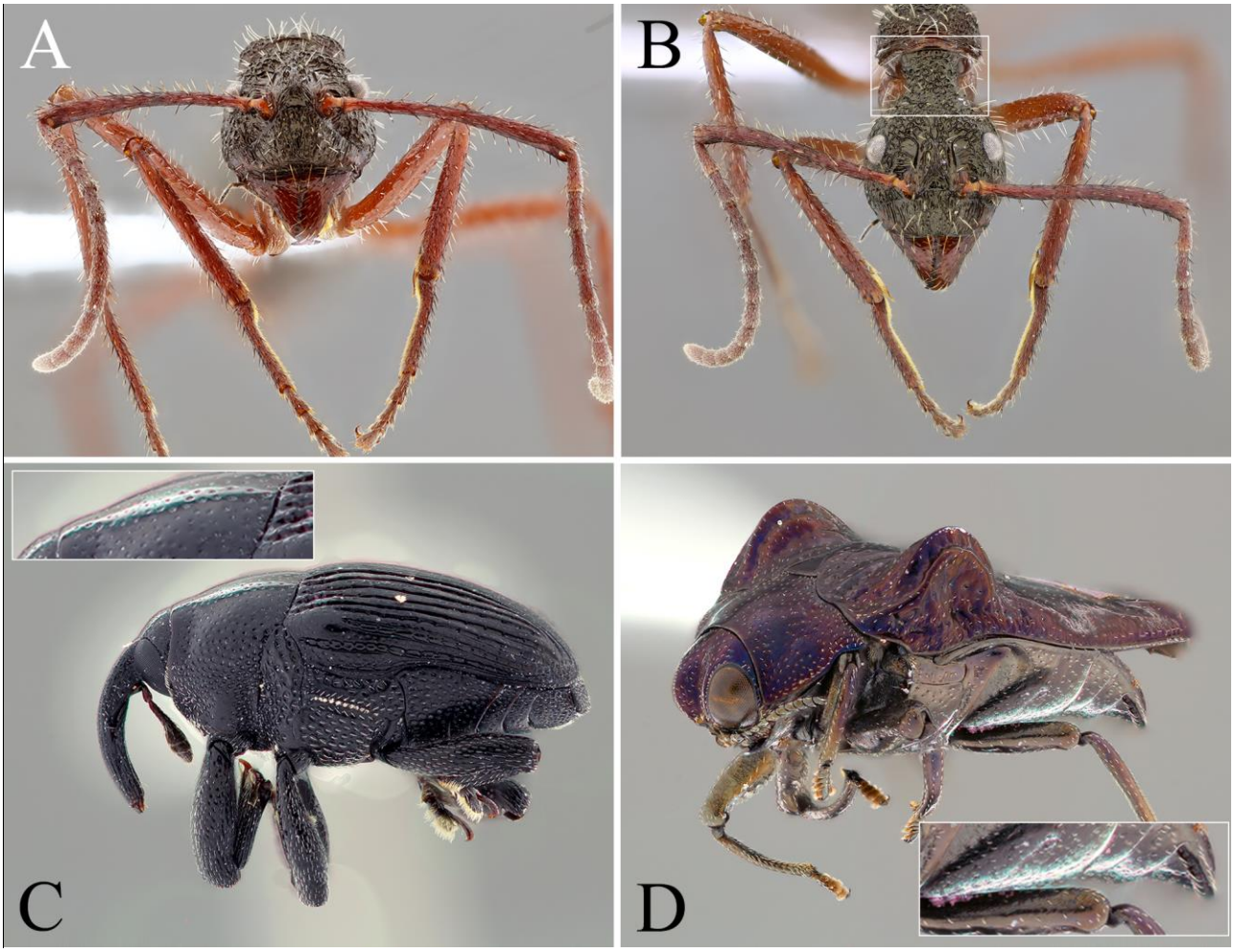

Figure 9. Errors in specimen positioning and image lighting due to improper use of the deflector ring. A and B) Head of Dolichoderus attelaboides, the image was intended to show the elongated vertex forming a tubular neck on the head; the first photo is from the front (so this tubular neck cannot be seen); C) Baridinae; D) Buprestidae. In C and B, overexposed areas appear as the light from the LED ring shines directly on the specimen because the deflector ring is not covering the specimen correctly. Photos: C. Flórez-V.

3.3. Image background

Various backgrounds can be used depending on the specimens' color, translucency, hairiness, structure, and sculpture. The most recommended for general use is a neutral grey (standard grey or 18 % grey), as it retains the colors, structures, and hairiness of the edges along the length of the insect while maintaining a correct contrast between the specimen and the background (Buffington *et al.*, 2005).

On the other hand, a white background can increase the light reaching the sensor at the risk of losing details at the edges of the specimen, such as pili or other structures. White also considerably increases the contrast in dark organisms or, on the contrary, decreases it in white or very light specimens. However, Bäumler *et al.* (2020) display some photographs successfully taken on a neutral white background. There are also structures where contrast is beneficial, such as wing venation in some groups of insects.

The black background is a good choice for very light or translucent insects (e.g., aquatic or immature arthropods, Figure 2E) and is unsuitable for dark specimens. Additionally, this type of background may require better lighting, so when using the dome system for bright specimens, some adjustments must be made to prevent dark or black reflections on the edges. This option has been widely used in publications as it gives images a more artistic look when appropriately used.

Various materials can be used for backgrounds (Figure 3 A, e). Grey molding clay is an option recommended for pin-mounted insects, as it allows the specimens to be manipulated and

arranged quickly (Buffington and Gates, 2008; Buffington *et al.*, 2005; Kawada and Buffington, 2016). In any case, these materials should be opaque and preferably homogeneous. At the same time, a distance must be maintained between the specimen and the background so the latter appears smooth.

As noted, using black backgrounds is more complex when using the dome lighting system since the light in this setup also hits the background and the specimen. Therefore, if you have a reflective material, you will get a dark blue background. To avoid this, you can use a black velvet-type fabric (Figure 3 A, e), which reduces reflection and maintains a relatively black coloration in the background (Figures 2E, 2F).

3.4. Photographing specimens in liquid

Photographing specimens in liquid has some additional complexities compared to dry specimens. Small Petri dishes can be used for the dome system, allowing the installation of a deflector ring. Two or more Petri dishes can be used opposite each other to separate the arthropod from the background and avoid textures in the latter (Figures 10A, 10B, 10C, 10D). The first Petri dish (or dishes) must be placed at the same level as the LED ring, so it must be supported by the background (Figures 10A, 10B). This dish supports the one facing up and housing the organism with the liquid (Figure 10C).

The described setup allows the specimen to be sufficiently high so the lens does not collide with the dome at high magnifications. In addition, it is recommended that only new Petri dishes be used for this purpose, thus avoiding scratches that could be seen in the photo and extending the editing process.

Maintaining the correct position of specimens in liquid is complex. For this purpose, various liquids that are more viscous than ethanol and allow adequate passage of light can be used. The most commonly used are those containing glycerin, such as glycerinated alcohol or gel, pure glycerin, or intimate lubricants. A pipette can deposit these liquids in the Petri dishes while gently expelling the liquid and avoiding filling the pipette with air to prevent inserting bubbles. Insects in ethanol must be placed in these liquids beforehand and thoroughly and repeatedly submerged (the specimen will tend to float) before taking the photograph so that the ethanol on the arthropod is thoroughly mixed with the other liquid.

Photographing insects in liquid also requires extra precautions to reduce vibrations throughout the system, and the effectiveness of the lighting is diminished because of the liquid. To overcome these problems, a higher ISO can keep the exposure time below 1/25. Here, it is even more critical to keep all equipment very clean since dust easily adheres to the liquid, and sometimes, it can appear at the same level as the specimen, making it more noticeable in the image. In addition, these tiny particles can remain in motion, generating strange patterns on the photograph after stacking, which represents a longer editing time.

3.5. Editing the final images

Other manuals, such as Bevilaqua (2020), provide detailed information on editing final images and producing plates for scientific publications, so this section is limited to giving some general indications.

Helicon Focus and Zerene Stacker allow making some corrections to the final image, so the "Retouch" option is crucial for some photographs with overlapping structures (Figure 6C). This option allows one to choose the series of captures where the structures closest to the observer are in focus and, using the "brush tool," leave only these areas in focus in the final image.

Before making any intervention, it is vital to remember that the images should not be arbitrarily altered and that editing is intended to correct aspects that do not modify shapes, structures, and color (beyond white balance corrections) (Bevilaqua, 2020). The lighting and color of the focal image, in particular, are two of the most essential elements in this process. This aspect can be simplified if, for example, there is good lighting management and white balance during photography. In addition, it is crucial to also make these adjustments in any copies of the original, preserving the latter intact.

In editing applications such as Adobe Photoshop, there are multiple options to correct these elements in Image > Adjustments. This software also has tools in Filter > Sharpen that considerably improve the definition of specific structures. Using tools such as the healing brush or the clone stamp,

dust or dirt particles can be cleaned up from the specimens in the final image.

Similarly, the background can be homogenized, erased, or corrected using various tools such as the Lasso (manually selecting the image area you want to leave) or the Magic Wand. The latter allows you to choose different tolerance levels (i.e., color similarity to selected pixels), which ultimately allows you to erase the background without affecting structures. Adobe Photoshop requires a license fee for its use, but there are also free options, such as GIMP (www.gimp.org/), with similar tools similar for image editing.

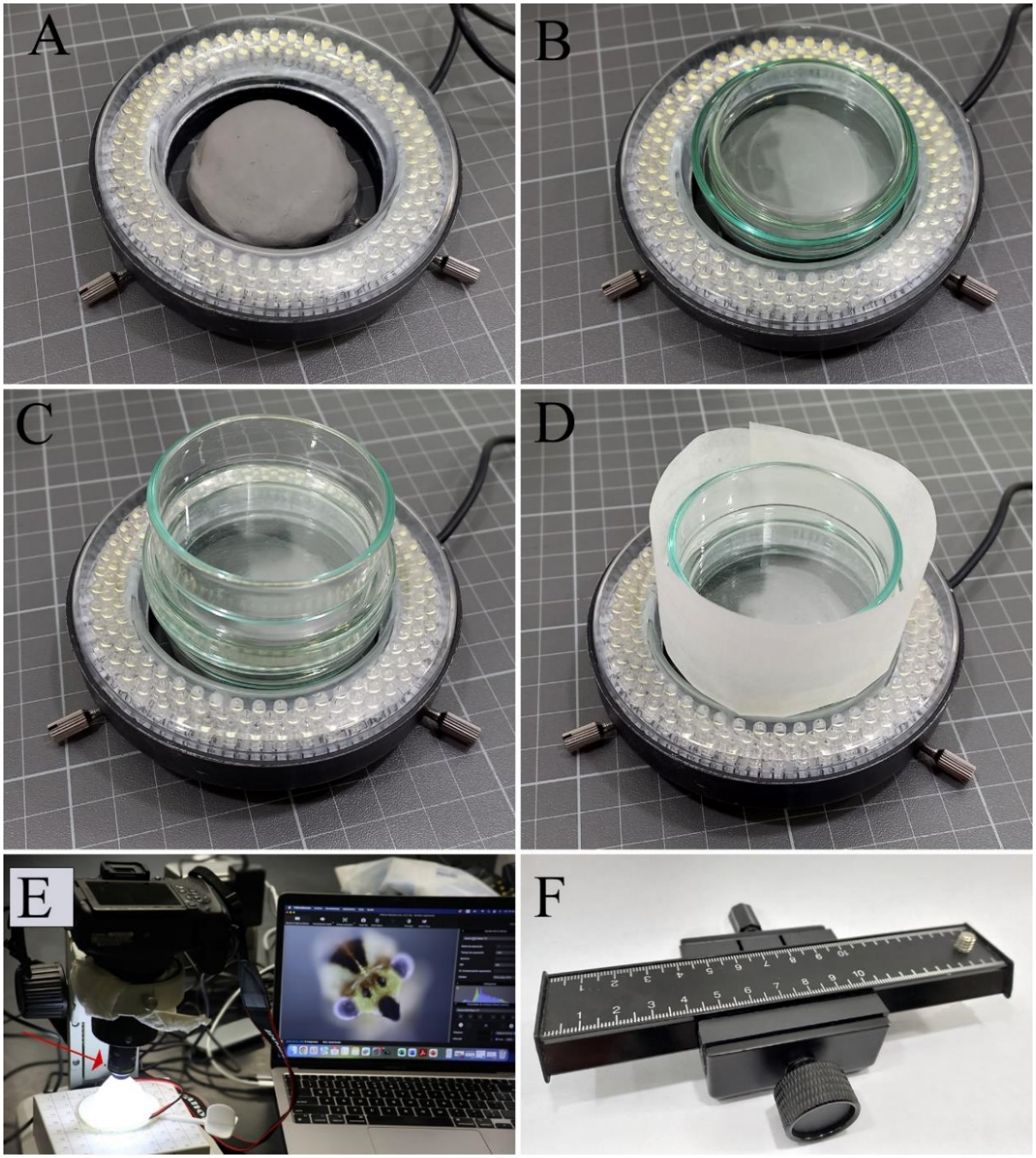

Figure 10. Variations related to the specimens' size or preservation method. A-D) Preparation of the Petri dishes photographing specimens in liquid. Figure D shows the use of the deflector ring in this configuration. E) The microscope objective is jerry-rigged to extension tubes. This system requires smaller domes and lights (as in Figure 3Ae). F) Rail moved using a crank. Photos: C. Flórez-V (E) and J. Cardona-Duque (A-D, F).

3.6. Post-processing of high-resolution digital images for different purposes

It is imperative to remember that the stacked images produced through these systems generally take up considerable amounts of digital storage (between 25 MB and 350 MB per image). The final file size may depend on factors such as the number of images used for the final image, camera settings like aperture and shutter speed, and the output file type (RAW, TIFF, or JPEG). Thus, it is essential to subsequently work with shareable files (i.e., through email or web server platforms) meant for different purposes, so storage strategies allowing smaller file sizes must be considered (Brecko and Mathys, 2020; Integrated Digitized Biocollections [iDigBio], 2023).

The steps optimized by the authors to reduce file size while maintaining efficient image quality using Adobe Photoshop software are described below. To this end, the following notation is used: tool usage is highlighted in bold and rounded type; tool name is indicated between brackets ("[ ]"); and keyboard shortcuts for Windows (Win) and MacOS (MAC) are in bold (between the symbols "< >").

The image obtained from Helicon Focus must have at least the following size and these parameters (it is recommended to keep the original files):

1) File type: TIFF.
2) Minimum dimensions: 39 cm x 30.05 cm.
3) Resolution: 300 dpi (figures 11A, 11B).

To check the image size and resolution, the Image Size dialog box must be activated with <Command + Alt + i> (MAC) or <Ctrl + Alt + i> (Win) (figures 11A, 11B). Here, you may change the color (see white balance). To save the image as TIFF, follow the path File, Save as, or by pressing <Shift + Command + s> (MAC) or <Shift + Ctrl + s> (Win), and name the file including relevant information (figure 7; e.g., "white balance corrected"), and select save it as TIFF (figures 11C, 11D, 11E, 12).

After saving the image in TIFF format, it can be cropped according to the presentation needs of the photograph (e.g., general view or morphological structure detail). To crop the image, select the Crop tool from the left menu or enter the command <C> (MAC and Win) (e.g. figures 11F, 11G).

It is advised to lower the file size by reducing the image size or resolution or by changing the file type (e.g., *JPEG extension) to generate high-resolution images that can be shared with colleagues or via image repositories. The Save for Web command (depending on the Photoshop version, it may be Export for Web) is recommended for saving an optimized image, following the path File, Export (figure 11H), or press <Shift + Command + Alt + s> (MAC) or <Shift + Ctrl + Alt + s> (Win). Select JPEG and Maximum quality (figure 11I).

It is also recommended that a medium-sized image (30 % of the original dimensions) be saved from the TIFF image. To do this, it is first necessary to reduce the image size by activating the Image size dialog box again and selecting Save as (Figures 11B, 11C, 11D, 11E). The size of this file will be approximately 10 % of the original file (Figures 12a, 12b).

Alternatively, it is recommended saving three additional optimized image files in *.jpeg, using the Save for web command (see previous paragraph): 1) a file with original dimensions saved in "Maximum" quality (this file size will be approximately 20 % of the original file size; figure 12c); 2) a file resized to 30 % (use the Image size command), saved in "Maximum" quality (this image will be approximately 2 % of the original file size; figure 12d); and 3) the same file as above (resized to 30 %), saved in "High" quality (this image will be approximately 0.5 % of the original file size; figure 12e). The latter is optimal for sharing by email and uploading to web pages as a thumbnail, which can be used as an icon on various dissemination platforms.

To safeguard copyright, it may be advisable that images published openly through biodiversity information systems or collection management platforms be watermarked with the logo of the biological collection or museum that generated them so that they are not used without the corresponding permissions, especially in lightweight output formats. In this way, users will be forced to formally request permission to use the image in high quality and give credits according to the terms defined in the collection management protocols. In addition, a return on use is guaranteed (for example, by increasing the taxonomic resolution of the specimens). Generating strategies for an adequate flow of images is vital in countries such as Colombia, where copyright (i.e., the moral rights of authorship over works) is protected by law, thus guaranteeing compliance with users' obligation to mention the author's name.

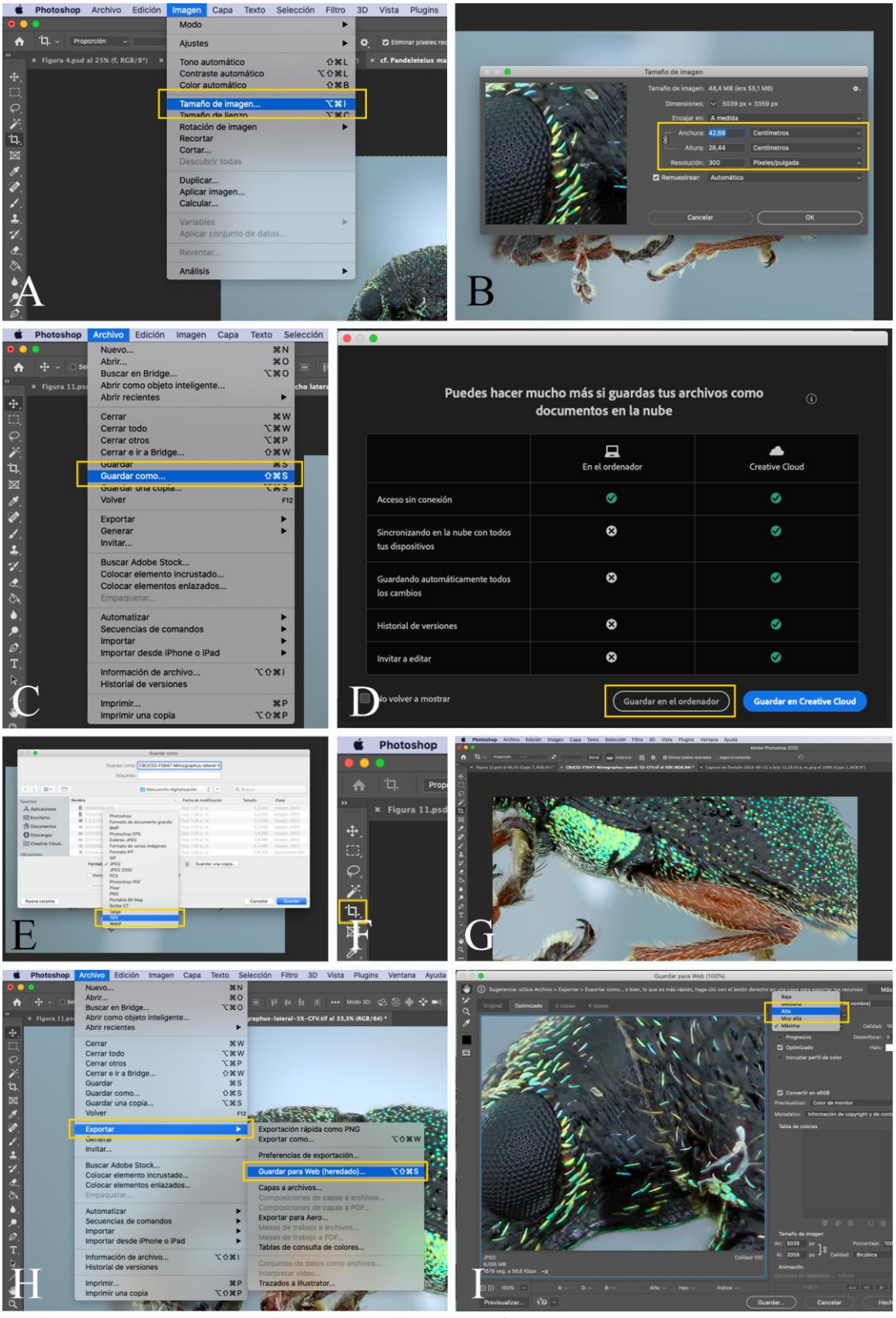

Figure 11. Steps for checking image size and saving images in different output formats. A-B) Panels are used to check and configure image size and resolution. C-E) Panels save files at their original resolution and size (or modified files in TIFF format). F-G) Panels are cropping the image according to the presentation needs of specific morphological details or structures. H-I) Panels are used to optimize file exporting in *.jpeg format. Photos: C. Flórez-V and J. Cardona-Duque.

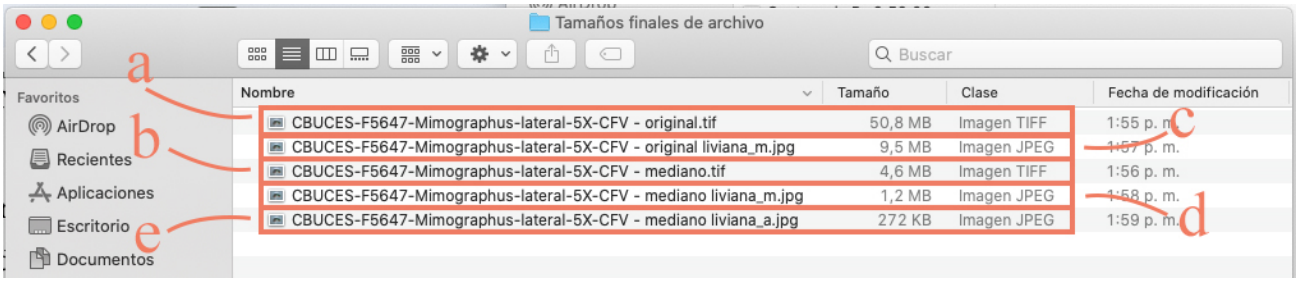

Figure 12. Output file sizes comparison, based on various purposes: (a) File in original size, resolution, and file type (*.tiff); (b) File resized at 30 % with 300 dpi resolution and original file type (*.tiff); (c) File with original size, 72 dpi resolution, and a lighter file type - "Maximum" export (*.jpeg); (d) File resized at 30 % with 72 dpi resolution, and a lighter file type (*.jpeg) - "Maximum" export; (e) File resized at 30 % with 72 dpi resolution, and a lighter file type (*.jpeg) - "High" export. Image: J. Cardona-Duque.

On the other hand, it is typical for the capture of original images to be interrupted by errors that force the process to start over again. Therefore, storing the photo sequences can take up as much or more space than the stacked images. For this reason, it is crucial to delete the original photographs not used in the stack; otherwise, excessive space can be taken up unnecessarily on the hard disk.

However, saving the image series is advisable if you need to generate the final image again, allowing you to use different stacking methods or applications. Depending on the type of image and whether the result is absent of significant errors after stacking, you can delete the sequence of captures.

3.7. Physical spaces for digitalization

It is crucial to consider the availability of an adequate physical space for setting up the photography system. In general, the site should reduce vibrations and movements that may affect the quality of the final images. It is recommended that the site not be close to elevators or roads where heavy vehicles travel and that the ground is stable (e.g., avoid raised floors, such as in containers). Likewise, there should not be constant foot traffic or unrelated activities occurring near the equipment. Experience shows these are a constant vibration source that can significantly hinder photography sessions (especially if the specimens are tiny or in liquid).

However, if these optimal conditions cannot be met, vibrations can be reduced by using sturdy tables and supports for the rail or camera. If the table with the photography system is shared with others, other activities should also be redistributed.

On the other hand, it is necessary to control external light, which can be reflected in the photographs. In the case of the dome system, this is not as impactful since the dome generally prevents such leaks; however, when using lenses with lower magnification (e.g., to take photographs of larger specimens), it is usually necessary to have a larger hole at the top of the dome (figure 4D), to facilitate the passage of external light. Likewise, some flash systems are exposed to more light, so sometimes it is necessary to create a dark space to prevent any light from entering (even from other lamps).

Finally, as with general collection care, the space must be kept dust-free, considering dust can stick to specimens and cover structures or adhere to (or enter) the camera, sensors, and lenses.

As mentioned in previous sections, the manual system (based on a stereomicroscope) or the automated system (using a motorized rail and mobile support) can be portable, implying that the system must be adapted to the space available for the collection at home or in the field.

3.8. Recommended equipment: specimen size and budget

Specimen size is crucial when choosing a photography system, mainly the magnification lens. The Canon MP-E 65 mm fixed-focus lens is recommended for insects between 2 and 25 mm, offering magnifications of 1X to 5X. For specimens larger than 25 mm, a similar setup can be used with a Canon EF 100 mm f/2.8L Macro IS USM lens, with some modifications to the illumination system.

Other lower-cost options (Table 3) include the Laowa 25 mm f/2.8 2.5-5X Ultra Macro lenses (replacing the Canon MP-E 65 mm) and the Laowa 60 mm f/2.8 2X Ultra-Macro (instead of

the Canon EF 100 mm). These work with Canon and other camera brands (specifically, Nikon and Sony). The downside to the Laowa 25 mm is that despite providing ample magnification, it can only be used on 2-10 mm specimens.

An alternative is manually constructing lenses from microscope objectives (Figure 10E) with magnifications greater than 5X. However, this adaptation requires many additional considerations regarding the quality and type of microscope objectives, camera adapters, and so on. Several resources on the web detail step-by-step how to make these lenses (extreme-macro.co.uk/microscope-objectives/).

As for cameras, Brecko and Mathys (2020) compared the image quality of full-frame cameras (with 35 mm sensors) and APS-C cameras (with sensors around 22 mm) using the focus stacking system with Canon MP-E 65 mm lens. Thus, researchers concluded that the image generated by the former setup is slightly better despite these cameras being much more expensive, so this tiny difference in quality does not justify preferring them over APS-C cameras (Brecko and Mathys, 2020).

With this system, the Canon MP-E 65 mm lens allows photographing specimens between 2 and 25 mm, visualizing many characteristics with adequate resolution, and preserving the specimen's colors, which is usually only possible with scanning electron microscopy (Figure 13). However, it is important to note that other higher magnification systems are needed to observe and describe structures much smaller than 2 mm in detail, such as lenses built with microscope objectives.

On the other hand, the manual system would be cheaper than the automated one, considering that collections may have dismountable stereoscopes for this use. In this way, you can employ supports from stereoscopes that are no longer in use to avoid moving the head and causing damage to the stereoscope's optics. There are also low-cost, non-automated rails that allow the camera to be moved with a crank (figure 10F), although the price may depend on the size of the movements.

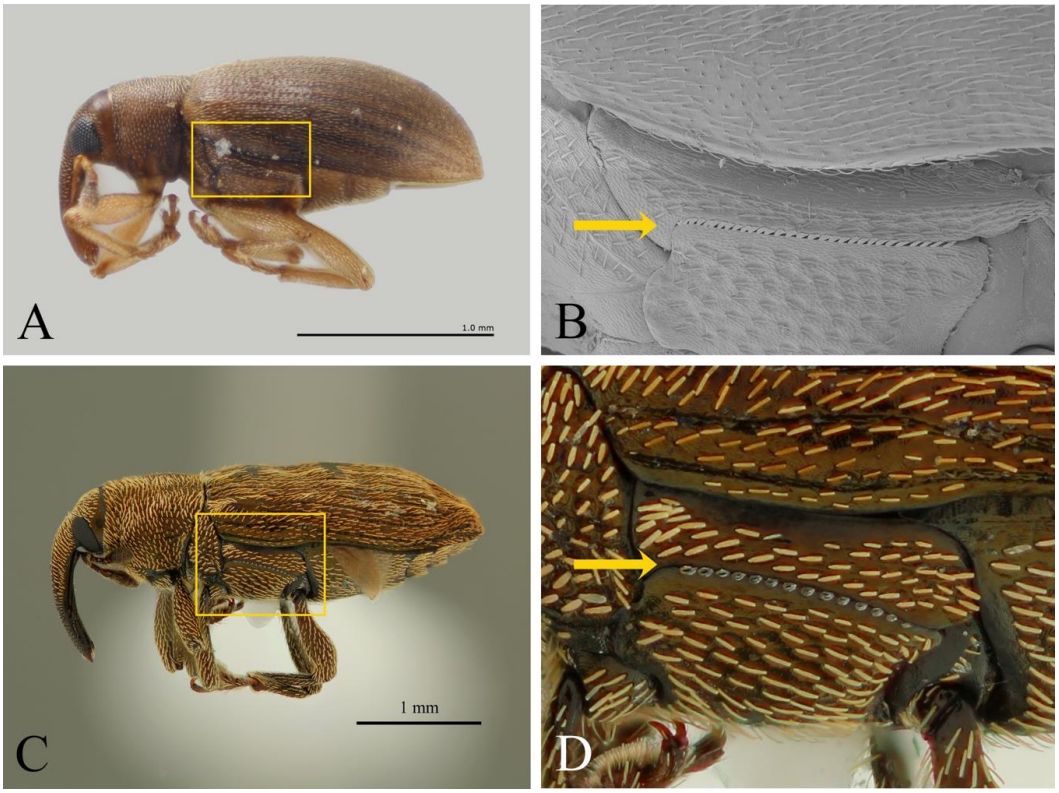

Figure 13. Detail of important morphological structures in the taxonomy of some weevil groups: A) *Azotoctla gomezi* (image generated with a high-cost system at INVCOL UPRM (UPRM Invertebrate Collection (INVCOL)); B) detail of the metepistern of *A. gomezi* where the sclerolepidia line is indicated (image generated with a scanning electron microscope); C) weevil of the subfamily Baridinae (image generated with the manual system proposed in this work); D) detail of the metepistern of Baridinae, where the sclerolepidia line is indicated. Note: Scanning electron microscopy can be used at higher magnification. It is even possible to see the sculpture of each of the scales. A comparison of these images is intended to show the level of detail achievable with this photography system at 5X magnification. Source: J. Cardona-Duque.

Among the motorized rails, two references from Cognysis StackShot are worth mentioning: the Macro Rail and the 3X Macro Rail, which differ from each other because the second allows controlling three motors that can move on three different axes, which is helpful if you plan to generate 3D models of the insects, in which case you can purchase other motors that can be configured from Helicon Remote or the rail console (cognisys-inc.com/learn-how-to/virtual-objects) (Doan and Nguyen, 2023). However, this 3X rail is more expensive than the Macro Rail (Table 3).

On the other hand, there is also the motorized rail from Wemacro (wemacro.com/), which is less expensive (table 3). Although there was no opportunity to use this option at the time of writing this article, the specifications given by the manufacturer, similar to those of StackShot, and some reviews on the internet suggest that this is an excellent alternative to reduce costs if you want to purchase a motorized rail. In addition, the company sells other products dedicated to macro photography (e.g., vertical support and camera adapters for microscope lenses). This rail can also be controlled with Helicon Remote, so most of the protocol detailed here can be applied equally to this reference.

Table 3. The cost of the equipment required for different arthropod photography systems in dollars (for comparative purposes in different Latin American countries) is described in the protocol. Note: The minimum recommended equipment is indicated with "R" (for around USD 2,659). *The StackShot 3X Macro Rail allows controlling two or three motors simultaneously, which helps generate 3D models (Doan and Nguyen, 2023). **License that can be used on four computers simultaneously.

Type of equipment	Equipment or software	Cost (USD)
Camera	Canon interchangeable lenses, APS-C (R) sensor	500 and up
Lenses: Specimens 2-25 mm	Canon MP-E 65 mm 1X-5X (R)/Venus Laowa 25 mm f/2.8 2.5-5X Ultra-Macro	1,049/399
Specimens 25 mm and up	Canon 100 mm/Laowa 60 mm f/2.8 2X Ultra-Macro	1,099/399
Automated system	Cognysis StackShot Rail: Macro Rail/3X Macro Rail*	579/879
	Rail support: portable, non-rugged/copy stand rugged, non-portable	50-100/500
	Wemacro rail/Wemacro vertical support	319/169
Manual system	Stereomicroscope support (R)	Recycled, ~100 and up
Lighting: dome, ring, or LED strips	AM Scope 144 LED Ring Light	105
	6,000k mouldable LED strip + 1(R) adapter	60
Flash	Macro Flash Canon Twin Lite MT24EX / Macro Flash Yongnuo YN24-EX TTL	989/189
Software license	Helicon Focus Premium Pack®** (R)	240 (in perpetuity)
	Zerene Stacker Professional Edition	280 (in perpetuity)
Computer	Computer with sufficient specifications to support Helicon Focus or Zerene Stacker (see manuals for both programs) and Photoshop (R)	600 and up
Image storage	Hard drive of at least 2 TB (R)	59 and up
Various materials	Dome, baffle ring, gray clay, USB cables (R)	~50

3.9. Cost and time of taking photos

The photography system described in this protocol is much cheaper. Based on Brecko *et al.*'s (2014) evaluation of stacking methods, it can offer results as good as stereoscopes with integrated cameras. As mentioned in the previous sections, some alternatives can suit different budgets, and despite requiring an initial investment, this system presents a good cost/benefit balance.

Table 3 presents an estimate of the equipment costs cited in the protocol. As can be seen, a digitizing station can generate high-resolution images with values between USD 2,400 (USD 2,659 when purchasing the equipment marked with R in Table 3) and USD 7,000. Mertens *et al.* (2017) reviewed other low-cost systems using compact digital cameras.

Another relevant factor when estimating costs is the time spent by the operators, which depends on the skill and training of the individuals and, to a certain extent, on the computer's processing capacity. In this sense, it is estimated that between the placement of the specimen in an appropriate position, the taking of the series of photographs, and the stacking of these, an individual trained in this system can generate an image in a time of 8-9 min (around six final images per hour), while someone without much experience can take 13-14 min (about four images per hour).

Other variables can affect the time it takes to generate an image: the size and shape of the specimen, magnification level, number of views per insect, specimen or lens changes, arthropod preparation methods, and system type (manual versus automated). It is advisable to pre-select specimens of a similar size and prepare them similarly during the same scanning session to reduce the time taking photographs.

4. Glossary

Alignment: process in which the same sequence of photos is adjusted into the same position. This way, the stacking programs find the areas focused in the same position throughout the sequence of photos.

Stacking: grouping photographs by focusing on different depth planes, where the resulting image is the union of the focused areas.

Layer of photos (= sequence of photos): individual photos with different focus planes that result after camera movement (figure 1A).

Stacked image (= output image or photo): final image produced by focus stacking a sequence of photos after processing through stacking software (Figure 1B, 2).

Number of shots (= number of shots): The number of photos the software will take on a programmed basis. It refers to the number of photos taken after selecting the nearest area 'A' and the farthest area 'B' and setting the distance of each step (Figure 4 g, j) while using a motorized rail. This refers to the number of photos that the software is set to take when using a stereo microscope stand (Figure 4 l).

Number of steps: The number of stops after defining the distance the rail will move between one photo and another (figure 5 g).

Step size: The distance (usually measured in fractions of a millimeter) the motorized rail moves between one photo to another (Figure 4 m).

Discussion

The generation of high-quality images has become an increasingly important tool in biological collections for academic, educational, and outreach purposes. The photography systems used for this purpose are becoming more affordable through the development of new low-cost alternatives (Bäumler *et al.*, 2020; Brecko *et al.*, 2014; Buffington *et al.*, 2005; Kawada and Buffington, 2016) and the recursive use of tools, supports and strategies for handling equipment and specimen lighting. Furthermore, the utility of the images generated far exceeds the required investments (Mertens *et al.*, 2017), and these identical setups have been used successfully in other biological groups (Bäumler *et al.*, 2020) and even in archaeological and geological collections (Brecko and Mathys, 2020).

When images are shared through biodiversity information systems and biological collection management platforms

(e.g., Symbiota), high-quality photographs can help collections advance in identifying their specimens. This is even more relevant in Latin American countries with high biodiversity, where many species remain to be identified and even described. Collaboration through these platforms also has the potential to increase joint work with specialists in other parts of the world and increase the visibility of collections, which also helps institutions manage resources.

Furthermore, beyond the strictly scientific field, photographs allow the creation of virtual collections that ultimately increase the social appropriation of knowledge by bringing information from these spaces to a broader audience. For example, the dissemination of these images through the social networks of institutions (e.g., Instagram CBUCES: www.instagram.com/cb_uces/) has shown that it can be instrumental in attracting different audiences and conveying the importance and work of biological collections (Lessard *et al.*, 2017).

High-quality stacked images and associated data support and drive replicable and verifiable work in anatomy, morphology, ecology, and phylogenetic systematics. Furthermore, in taxonomy, this type of material often facilitates the description of external morphological characteristics.

However, it must be acknowledged that the increasing digitization of biological collections poses a challenge to managing the storage of images, the data produced by the stacking process, the tracking of metadata created, and their remote online access (Brecko *et al.*, 2014), which has motivated the foundation of several repositories that allow file storage (e.g., DISSCo: https://www.dissco.eu/; Figshare: figshare.com; Dryad: datadryad.org; Zenodo: zenodo.org; The Open Science Framework: osf.io).

Images can also be hosted on specialized platforms such as MorphoBank (morphobank.org), MorphBank (www.morphbank.net) or TaxonWorks (taxonworks.org), or databases for particular taxa such as AntWeb (antweb.org) or Orthoptera Species File (orthoptera.speciesfile.org) or on more general-purpose databases such as Symbiota portals (e.g., SCAN [Symbiota Collections of Arthropods Network]: scan-bugs.org). They can also be published on local biological collection websites such as CBUCES (demo version: demo-cbucesdata.pythonanywhere.com) or CEUA (ceua.pythonanywhere.com), linking them to datasets on biodiversity information aggregators such as GBIF (Global Biodiversity Information Facility: gbif.org).

These databases and platforms have become more valuable over time due to the increasing accessibility and power of computing technology (Schuh *et al.*, 2010). The level of sophistication in this field has led to multiple applications, including the potential use of deep learning methods to detect species in insect monitoring (Høye *et al.*, 2021).

This work has focused on the capture of photographs and their subsequent handling. However, there are previous stages that must be taken into consideration. For example, it is essential that collections prioritize the material to be photographed and that assembly processes are carried out rigorously. These decisions can be made according to the nomenclatural importance of the material (type specimens), the taxonomic-morphological representativeness (e.g., population variability), or the geographic (e.g., endemisms), or the need to preserve records of characteristics mutable over time, such as color. Sometimes, it may even be decided to select spectacular specimens that amaze general audiences (annex 3) or arouse high interest among researchers. In this way, collections can optimize the time of the personnel in charge of digitization, the resources invested, and the academic, scientific, and dissemination impact.

Other additional tasks that should be judiciously planned within a protocol for digitizing biological collections are the generation of labels associated with specimen photographs and the assignment of a (unique) catalog number, which facilitates sharing and linking image information to species lists and occurrence data published through platforms such as GBIF, enhancing the use of visual records (e.g., https://www.gbif.org/occurrence/3329184428). To this end, protocols such as the one proposed by Blagoderov *et al.* (2012) for mass digitization of labels can be applied, or processes of label photography can be added, complemented with automatic text recognition systems, which allow automation of the process.

The stacking system described here can be applied to create 3D models (Doan and Nguyen, 2023) using relatively

low-cost equipment, including the use of motorized rails (such as the StackShot 3X with an additional rail) or manual ones, which facilitate the rotation of the specimens (or the camera) in different predetermined directions. Furthermore, the images generated can have further uses in comparative biology through protocols, tools, and software that allow other types of analysis, such as morphometric analysis (Porto *et al.*, 2021; Shui *et al.*, 2023).

Finally, returning to the initial idea that led to this work's conceptualization, structuring, and design is crucial. After several years of experience, the authors learned, refined, and implemented creative solutions to generate digital images of arthropod specimens in biological collections; it was possible to recognize that the initial austere investment in relatively low-cost equipment (an inexpensive camera, MP-E 65 mm lens, a LED ring, a stereomicroscope stand used in teaching, a dome built with a hemispherical plastic funnel and the open license Combine ZP application) ultimately allowed a significant increase in the quality of generated images.

This method gave new meaning to the power of images since the improvement in their quality allowed the digitalization of a large number of specimens (in fact, with an almost maddening drive to generate images of everything at the beginning, even in a disorderly manner and without establishing a system for file management and nomenclature), visualizing previously unnoticed structures, and proposing functional morphology hypotheses. Of course, these advances also opened the door for the authors to science dissemination in entomology, with the desire to share a small amount of the beauty in morphological diversity that became visible. Thus, the hope is that this protocol will increase the digitalization of specimens in Latin American biological collections.

Conflict of interest

The authors have no conflict of interest.

Authors' contribution

Camilo Flórez-V. and Juliana Cardona-Duque: conceptualization, data collection, image generation, and document writing.
Larry Jiménez-Ferbans: data collection and document writing.

Agradecimientos

We would like to thank the biological collections at Universidad CES (CBUCES) and Universidad del Magdalena (CBUMAG), as well as the Frost Entomological Museum (Pennsylvania State University), for providing specimens and space. We would also like to thank Ricardo Kawada, Gabriel Biffi, and Olivia Evangelista for their enthusiasm in introducing Camilo Flórez to insect specimens digitization; Tate Nathan Geiger for help in building the 3D printed dome; Diana María Carmona from the laboratory area of Universidad CES, for providing space and equipment in the early stages of the digitization trials. We would also like to thank Ana Ospina Montoya for her support in designing and constructing LED triangles and miniature domes and Camilo Chica for his support in the final editing of the tutorial videos. Finally, we thank Juan Pablo Montoya Cano and Juan Camilo Arredondo for their empirical feedback on various processes for technical adjustments to the digitalization systems at CBUCES. This work was partially funded by GBIF-BID National Biodiversity Data Mobilization Grants (project BID-CA2020-045-NAC), the University of Magdalena, and the CES University.

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