Notas

Detection of human DNA in larvae and adults secretions of *Calliphora vicina* (Diptera: Calliphoridae) fed on human liver

Detección de ADN humano en larvas y secreciones de adultos de *Calliphora vicina* (Diptera: Calliphoridae) alimentados con hígado humano

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Abstract: Through the analysis of DNA extracted from the gut contents of larvae known to consume human tissue, a methodology emerges for determining the source of their sustenance. In this study, human liver was provided as a food source to larvae and adults of *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae). Emphasis was primarily placed on evaluating the quality and persistence of human genetic material present in the gut of larvae as well as in the secretions of adults that emerged from larvae fed on human liver. The findings revealed that viable genetic material appropriate for human identification could be discerned only within the larvae gut, even after a two-hour fasting period.
Keywords: Blowflies, Gut content, Human DNA, STR analysis.

Resumen: A través del análisis de ADN extraído del contenido intestinal de larvas que consumen tejido humano, surge una metodología para determinar la fuente de alimento. En esta investigación, se proporcionó hígado humano como alimento a larvas y adultos de *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae). El énfasis se centró principalmente en evaluar la calidad y persistencia del material genético humano presente en el intestino de las larvas, así como en las secreciones de los adultos que emergieron de larvas alimentadas con hígado humano. Los hallazgos revelaron que solo dentro del intestino de las larvas se puede discernir material genético humano viable, proveniente de la fuente de alimento, incluso después de un período de ayuno de dos horas.
Palabras clave: ADN humano, Análisis STR, Contenido estomacal, Moscas azules.

Forensic entomology is a branch of biology that deals with the study of insects and other arthropods present in a corpse in the legal and forensic field for investigative purposes. One methodology used to estimate the postmortem interval (PMI) involves examining the development of dipterans within cadaveric remains. This estimation often relies on assessing the oldest collected larvae obtained from a deceased body (Villet, 2010). If it is possible to find the oldest stage that developed in the corpse and establish its age, in hours or days, it is possible to know when the body was first exposed to the action of insects (Centeno, 2000; Magaña, 2001; Centeno et al., 2002).

Cases arise where the substrate in which the larvae developed cannot be identified, either due to the absence of a corpse or the presence of other food sources (Wells et al., 2001). In such scenarios, the genetic material within the larval digestive tract becomes a valuable resource for determining the identity of the cadaver on which they fed (Carvalho et al., 2005). Some preliminary research claims that mitochondrial DNA was detected in the larval intestine of *Protophormia terraenovae* Robineau-Desvoidy (Diptera: Calliphoridae) larvae, even up to four days after consuming human tissue (Njau et al., 2016).

Some adult dipterans can act as vectors for human DNA by depositing fecal or regurgitated stains after feeding on blood or semen (Durdle et al., 2013a). Referred to as "fly artifacts," these marks have been found to contain significant quantities of human biological material in *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae) adults, thereby enabling the generation of complete or partial DNA profiles (Eckert & James, 1998; Durdle et al., 2009,2011, 2013b).

The main objectives of this research are to determine whether identifiable human DNA can be extracted from *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) larvae digestive tract and from adult artifacts after they have consumed human liver, by identifying autosomal markers, and to establish the minimum residence time of human genetic material within the larval intestine after ingestion, by comparing two different techniques employed to extract the intestine contents.

We maintained a colony of *C. vicina* within an incubator, with 22 °C of temperature, a 12L:12D photoperiod, and access to a diet of sugar and human liver *ad libitum.* All adult flies were fed with human liver before oviposition. The larvae were also fed on human liver tissue and completed their developmental stages on this substrate. The emerging adults from the pupae also continued their development feeding on human liver.

The human liver tissue, derived from a single individual, was obtained from anatomical samples during an autopsy. The donor was a 35-year-old Caucasian male who died from a stab wound, and the tissue was fresh at the time of the autopsy. The liver sample was provided to us as discarded material by personnel from the Forensic

Medical Corps of the city of San Carlos de Bariloche. Throughout the entire process, the sample was kept at a -18 °C of temperature. A tissue sample, known as an indubitable sample, was taken to obtain the genetic profile of the donor. This sample was used to compare with the genetic profiles obtained from the larvae intestine. The experimental protocol followed the standards of quality and safety for the postmortem tissue (Directive 2004/23/EC) and was approved by the Ethics Committee of the Hospital Zonal Dr. Ramón Carrillo.

Fly artifacts from adults fed exclusively on human liver were analyzed without differentiating between fecal or regurgitated stains. Excretions from these adult flies were collected immediately after emerging from the puparium, and this specific excretion is referred to as meconium. These adults originated from larvae that had completed their development on human liver. This study investigates whether the meconium contains residues from the human tissue they consumed during their larval stage.

We used two separate rearing chambers: one containing the pupae about to emerge and another housing adult flies with access to food. To gather artifacts, we located FTA (Flinders Technology Associates) papers within the rearing chamber of 40 adult specimens for a 24-hour duration while providing human liver as food. This timeframe enabled the direct excretion and regurgitation of the specimens onto the FTA papers (Durdle et al., 2009, 2011, 2013a, b). To collect meconium, FTA papers were placed within the breeding chambers during the same allotted time while 40 newly emerged adults from puparium were isolated without any food source.

Given the susceptibility of human DNA degradation within larval intestines, we took measures to establish an optimal residence time by subjecting the larvae to different fasting durations prior to dissections. The first and second instar larvae were subjected to a no fast condition (0 minutes), while the feeding third instar larvae underwent a fasting period of up to 120 minutes. The feeding third instar larvae were allowed to feed and then subjected to fasting periods. Samples were taken at 30, 60, 90, 120, and 150 minutes of fasting. After each fasting period, the larvae were dissected.

The larvae were in constant contact with the tissue and it was necessary to guarantee asepsis before performing the extraction of the intestine contents. We washed the larvae in three successive baths of 200 ml of distilled water (Eckert & James, 1998; Carvalho et al., 2005). We sterilized under flame Petri dishes and instruments (Linville & Wells, 2002). To prevent contamination of the samples, we implemented stringent biosafety measures within the laboratory, including the use of personal protective equipment, such as gloves, lab coats, and face masks. The work was conducted in a special room with restricted access to unauthorized personnel. Additionally, we sent biological samples from all project participants to screen for potential human cross-contamination.

For the extraction from the larvae intestine, we employed two distinct techniques and process two larvae for each instar:

Dissection. Dissections were conducted during the third instar larvae. Prior to dissection, each larva was briefly immersed in liquid nitrogen for three seconds to freeze the cuticle and facilitate precise cutting. Subsequently, a ventral incision was carefully made in the cuticle, following a posteroanterior direction, using a No. 4 scalpel. This incision allowed for the exposure of the intestine. The intestine was then extracted using needles and placed onto FTA papers to prepare for subsequent analysis (Linville & Wells, 2002; Butler, 2009).

Compression. The compression technique was applied to the first instar, second instar, feeding third instar, and post-feeding third instar larvae. This method entailed meticulous compression of each larva within FTA paper.

We allowed all FTA papers to air dry. These dried samples were stored within paper envelopes and then placed inside a closed box to protect them from contamination, preparing them for the subsequent DNA extraction phase.

The samples were analyzed by the laboratory of Huellas Digitales Genéticas de la Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires. The DNA laboratory participates in quality controls of GHEP-ISFG and SAGF (Spanish and Portuguese Speaking Group of the International Society of Forensic Genetics and Argentine Society of Forensic Genetics).

For the process of DNA extraction, we employed the automated extraction system Maxwell® 16 system from Promega Corporation. Subsequently, DNA quantification was done with real-time PCR using the Rotor Gene 6000 platform by Corbett Life Science (Australia). This quantification process involved the application of the Plexor HY system (Promega Corporation) and the Amel-Y (Ginart et al., 2016) duplex.

To amplify the 27 autosomal markers, we utilized the PowerPlex Fusion 6C system from Promega Corporation. The amplification took place within a Gene Amp 9700 PCR system from Applied Biosystems in Foster City, USA, in strict adherence to the instructions of the manufacturer. The resulting amplification products were subjected to analysis on an ABI 3500 genetic analyzer from Applied Biosystems in Foster City, USA.

For each genetic marker we calculate a numerical value, known as the Identity Index (ID), to gauge the strength of genetic evidence. The ID is derived from the calculation of the Likelihood Ratio (LR). The total ID is determined by multiplying the individual IDs together, following the product rule (Butler, 2014). A higher ID, particularly values greater than 1000, indicates stronger genetic evidence linking the suspect's samples to the processed samples (Evett et al., 2000).

We evaluated 24 DNA samples derived from dissections, compressions, artifacts and meconium. Notably, positive outcomes were obtained only through the implementation of the larval compression technique.

The results we obtained indicate that compressing the larvae is a more efficient method for obtaining DNA, and it does not compromise the integrity of the DNA within the intestine. Indeed, dissection of the intestine was only feasible in third instar larvae due to their size, which allowed for accurate dissection without material loss. In the case of earlier instar larvae, we were unable to achieve successful results due to the smaller intestine size.

The dissection of the larval intestine demands specialized expertise in larval anatomy and proficient handling of entomological instruments. Therefore, having personnel trained in entomological laboratory management is crucial for the safe and effective execution of this technique (Linville et al., 2004). Although the use of liquid nitrogen eases the difficulties of larval intestine extraction, this technique was not effective in obtaining human DNA. However, the technique of compressing the larva between FTA papers offers a simpler approach to achieve successful DNA extraction, without requiring specialized personnel, and being more efficient in sample collection. Through this approach, we successfully imprinted the entirety of the DNA content onto the paper, capturing not only the genetic material of the larva itself but also the contents of the intestine.

We obtained a singular genetic profile from the undoubted sample corresponding to a male individual. Analysis of the larval samples yielded partial genetic profiles that were consistent with the profile obtained from the undoubted sample. Notably, we successfully established a positive ID for each of the feeding instar larval, as shown in Table I.

TABLE I.

Identity index calculated for each positive DNA sample.

TABLE I. Identity index calculated for each positive DNA sample.

Larva	Identity index
first instar	7.6×10^{7}
second instar	9.2×10^{16}
third instar	2.4×10^{12}

We derived partial DNA profiles from all actively feeding instars. Figure 1 visually illustrates that the second instar larvae displayed the highest concentration of DNA. We observed a high concentration and positive identification in the feeding instars. The second instar larvae demonstrate higher levels of partial DNA content compared to the first instar and feeding third instar larvae. This disparity can be attributed to several factors. The first instar larvae possess a little

intestine with limited food retention capacity, which can contribute to reduced DNA content. In the case of feeding third instar larvae, their larger size provides them with the potential to retain more food; however, this instar also boasts a higher concentration of nucleases that can lead to DNA degradation (Hobson, 1931; Arnott & Turner, 2008).

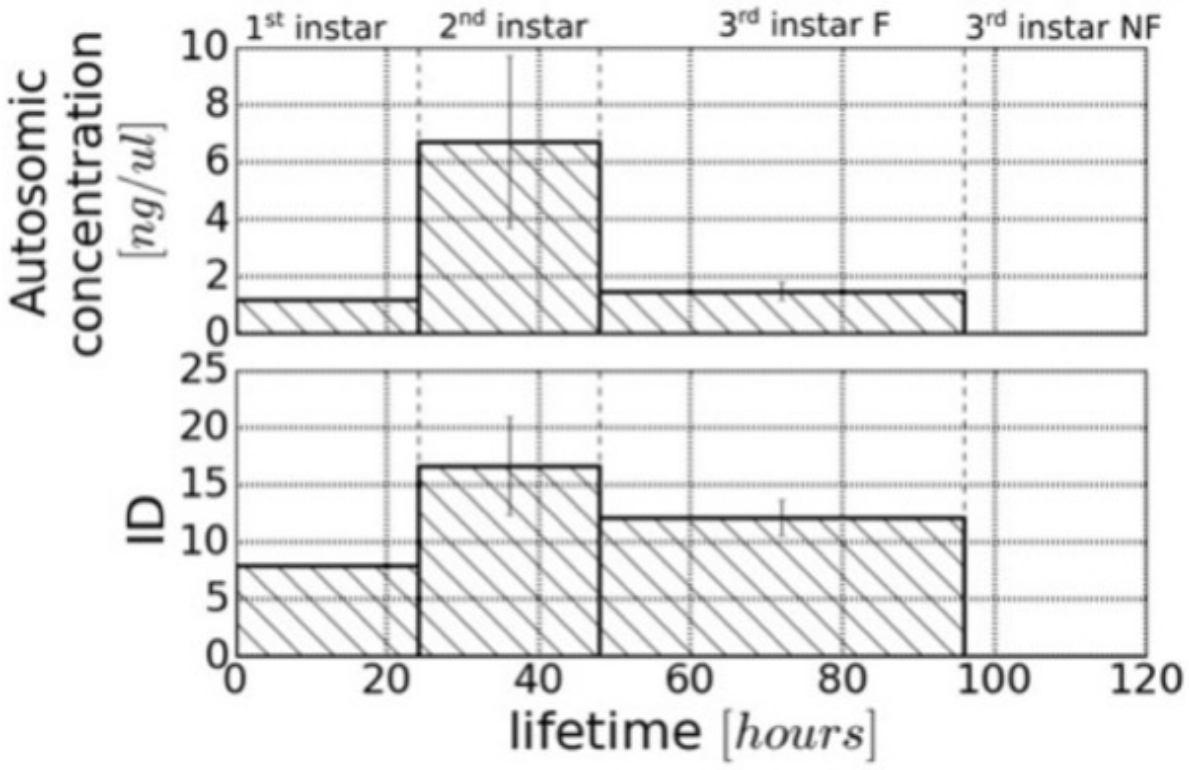

Fig. 1.

Amount of DNA and Identity Index (ID) in each larval instar.

On the horizontal axis we represent the life time, expressed in hours. On the lower vertical axis, we show the logarithm of the ID and on the upper vertical axis we show the concentration of autosomal markers found in each instar. The 1st instar, 2nd instar, and 3rd instar correspond to the larval stage. The 3rd instar F indicates the feeding stage, while the 3rd instar NF indicates the non-feeding stage.

Figure 2 illustrates that under fasting conditions, the maximum duration allowing for reliable identity establishment is two hours (ID= 3.8×10^{22}).

Our notable finding is that two hours is the maximum duration of fasting for larvae, allowing reliable establishment of identity. This effect is attributed to the swift action of nucleases, which contribute to DNA degradation (Hobson, 1931). Interestingly, our results diverge from those published by Njau et al. (2016). In their study, DNA quantification was conducted on third instar larvae of *P. terraenovae* following a four-day period of abstinence (Njau et al., 2016).

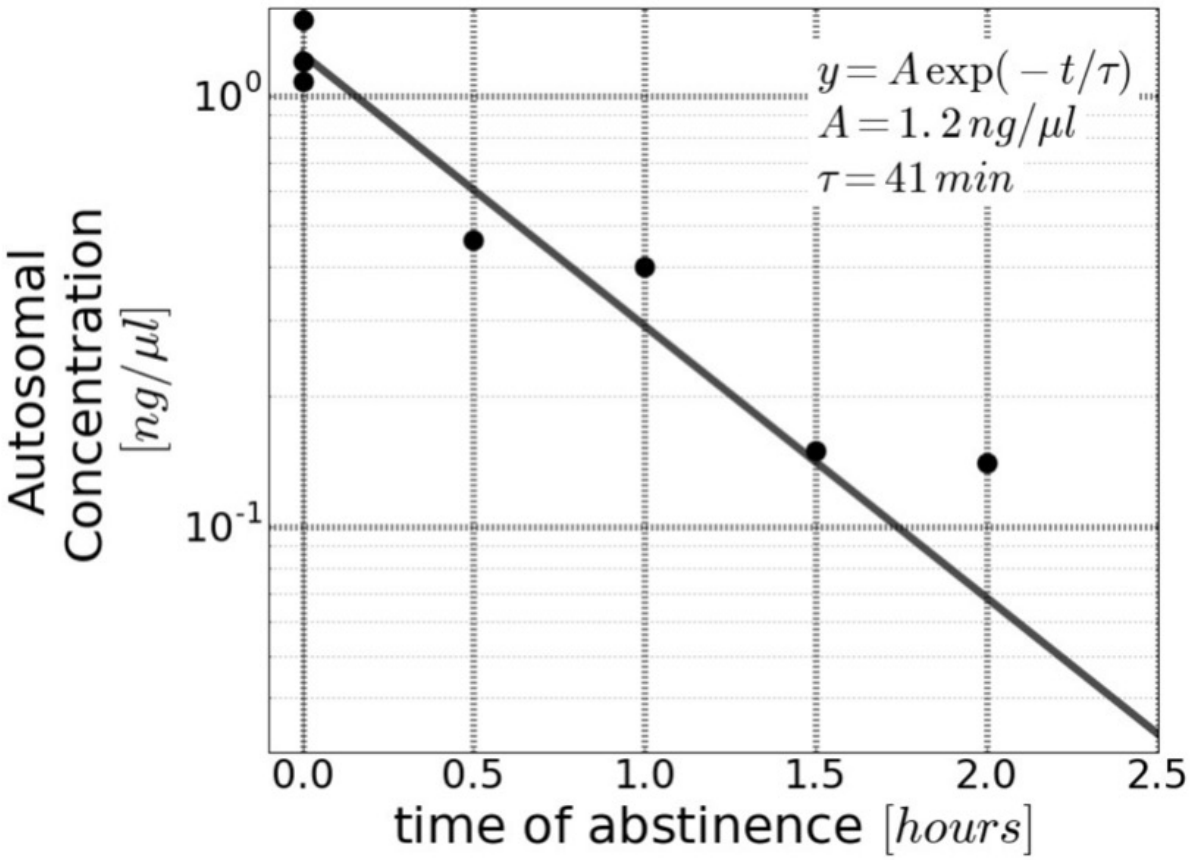

Fig. 2.

DNA survival after a fasting period.

On the horizontal axis, we represent the fasting period of third instar larvae, and on the vertical axis, we show the autosomal concentration (ng/µl). We find that the maximum time to establish identity is 2 hours (ID = $3.8x10^{22}$).

Despite our analysis of artifact samples, we regrettably did not detect any DNA traces within them. These results are in contrast with those published by Durdle et al. (2009, 2011, 2013a, b). This unfavorable outcome could potentially be attributed to either the relatively low concentration of fly stool deposited on the FTA paper or the divergence in sample composition, as we employed human liver rather than the semen or blood utilized by the referenced authors.

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