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USE OF TRANSPONDERS FOR INDIVIDUAL MARKING OF *UNIO CRASSUS* PHILIPSSON, 1788 (BIVALVIA: UNIONIDAE) IN MOUNTAIN RIVERS

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Abstract: Usefulness of Passive Integrated Transponder (PIT tag) technology for observation of behaviour and life history of endangered *Unio crassus* was tested in two Polish mountain rivers. Dispersion of PIT marked individuals from the place of release did not exceed 3 m. The detection of marked individuals on the rocky bottom was very low (13–39%) and decreasing with time. Ca. 1/3 of implanted PIT tags were rejected, usually within two weeks after implanting; later the rejection did not occur as the PIT tag became fixed in the nacre (very thin on the PIT tag surface adjacent to the flesh, thick with additional fixing structures adjacent to the shell). Nevertheless, 33 tags were detected after three years, some in live individuals which were more numerous on the soft sediment bank (n=12) than on the rocky bottom (n=6). The influence of electromagnetic field on the detection of PIT tags, the possible causes of the tag rejection and mechanisms of tag retention are discussed. It is suggested that PIT tags could be useful as a method of durable individual marking but less suitable for detecting and/or locating the mussels. Controlling of possible tags rejection is indispensable.

Key words: RIFD chip, PIT tags, individual marking, individual identification, mussels

INTRODUCTION

Individual marking is one of the basic tools in contemporary ecology. It enables the researcher to follow individual life histories and even to collect lifetime information leading to robust conclusions on life history traits and population demography (Clutton-Brock & Sheldon 2010). Individual marking makes it possible to study behaviour (e.g. for *U. crassus* see Zając & Zając 2011) and to trace subsequent fate of different individuals. Without the so-called longitudinal data, the estimation of life history traits and basic demographic parameters of a population is always equivocal and can be questioned (Nussey et al. 2008).

A number of different tools are used for individual marking of molluscs. At first sight, marking seems to be very easy, considering the firm character of the shell, which can be marked and usually preserved after the animal's death. The shell can be individually marked using symbols/numbers or alphanumerical codes. These can be scratched in the periostracum, or painted on with various more or less permanent paints, including oil markers, permanent markers, various glues (e.g. those hardened by water, the so-called "superglues", which are very firm and become white once hardened); numbered tags can be glued on (see: Hartman et al. 2016, for review) or fixed with wire on the shell perforated for this purpose. However, in my experience all these methods have their restrictions: scratching is tedious and may lead to weakening, erosion or even perforation of the shell, whereas marking with paints or glues requires drying of the shell surface and/or cleaning it. For both methods the mussels have to be removed from the water for longer periods, which may cause a decline in their performance. The external marks can be worn by the sediment, and all the different types can be difficult to read after some time, when they become covered by precipitates from the water (usually black and non-transparent). The periostracum on the oldest parts of the shell can be worn or even eroded by the

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sediment, which can cause further dissolution or mechanical damage of the marked spots. Furthermore, if the shell is broken into pieces, or burrowed within the sediment, or overgrown or in any way covered by organic matter or vegetation (e.g. periphyton), it can be easily overlooked or misidentified.

One of the best possible ways to follow the individual history is to use radio transmitters that precisely locate the marked individuals (Zając & Zając 2011). However, in the case of most mussels or snails this technique has a serious constraint: the radio transmitter has to be small, and thus its battery must also be small. This significantly restricts its operating time to weeks or months – far too short in relation to the expected lifetime of the studied individuals, which can reach dozens of years (e.g. in large freshwater mussels). Due to the relatively large size of the transmitter, it is usually impossible to mark young individuals whose life histories are most interesting from the scientific point of view or which are considered to be more important in restoration programmes (Hua et al. 2015). The technique is also expensive and, moreover, some radio transmitters, especially if their size is kept to a minimum, are vulnerable to mechanical damage or water leakage.

The lifetime and size restrictions can be overcome with the use of transmitters that do not require energy supply. It would be the best solution to implant such a transmitter inside the shell, where it would be safe as long as the animal is alive and even for a long time after its death, thus possibly allowing for the collection of reliable data on the features and mortality of the mussels. This opportunity is offered by PIT tags (Gibbons & Andrews 2004; also called: "passive induced transponders" or "transponders" or "radio frequency identification (RFID) tags"; in Polish: "znaczniki scalakowe"). Theoretically, the ideal solution for PIT tag application is to insert it between the mantle and the shell, as in pearl cultures, assuming that the mantle would anyway produce nacre to cover the tag and fix it to the shell. However, application of the tags generates some questions: how durable is this solution? Can the transponder be incorporated within the nacre layer, becoming a firm part of the shell? In such a case the transponder will be fixed to the shell almost forever, until it breaks into pieces small enough to expose the PIT tag to damage. For how long can the tag operate and be readable from the outside? What happens when the mussel dies?

MATERIAL AND METHODS

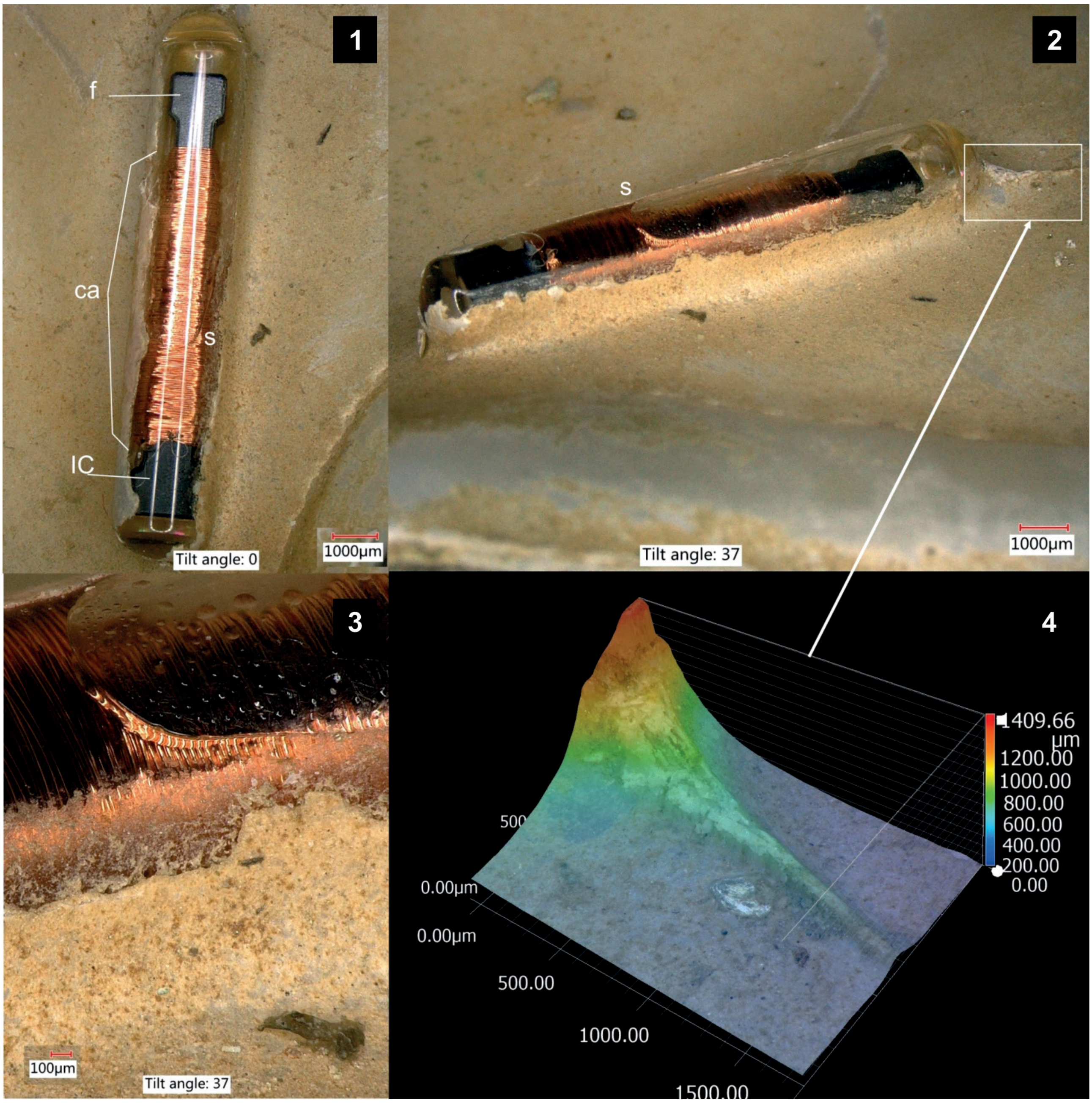
The study object was the thick-shelled river mussel *Unio crassus* Philipsson, 1788, a species of large body size and long lifespan (8–50 years), dioecious, and a short-term brooder. Its short-lived larvae need a fish host to complete their development. The species inhabits flowing waters, both montane and lowland. Despite its threatened status it is still insufficiently studied (Lopes-Lima et al. 2017).

PIT TAG TECHNOLOGY

The PIT tag contains an integrated circuit (IC), which is programmed by the manufacturer with a unique code. The idea is that (Figs 1–4) the tag's miniature coil antenna, enclosed within a glass tube, can be induced with an electromagnetic field produced externally by a large-size powerful antenna. The induced miniature antenna will then generate electric power, which will activate the microchip, which in turn will emit a series of impulses, already coded within it, using micro antenna. This signal will be received by the inducing antenna attached to a receiver, which filters the signal, amplifies it, and decodes it (from a series of 01 bits coded in the impulses) to alphanumerical code, and shows it on the receiver's screen. According to the laws of physics, the maximum strength of electromagnetic field is in the plane of the loop (the shape of the lines of electromagnetic field is symmetrical below and above the plane of the loop of the inducing antenna, and thus the positions shown above the antenna would be the same as those below it). The field decreases very quickly with the distance from the loop – the range of tag detection is usually less than 50 cm in the air. The induction efficiency depends on the position of the tag's micro antenna in relation to the electromagnetic field lines generated by the large antenna (Figs 5–7).

HPT12 PIT tags (12.5 mm) of Biomark Co. (USA) were used. The accessories, including a receiver and two types of loop antennas: a standard antenna with a power supply from the receiver and a strong field antenna which needs an additional power supply and special carrying equipment due to its weight, were tested. Power was supplied by extra rechargeable batteries.

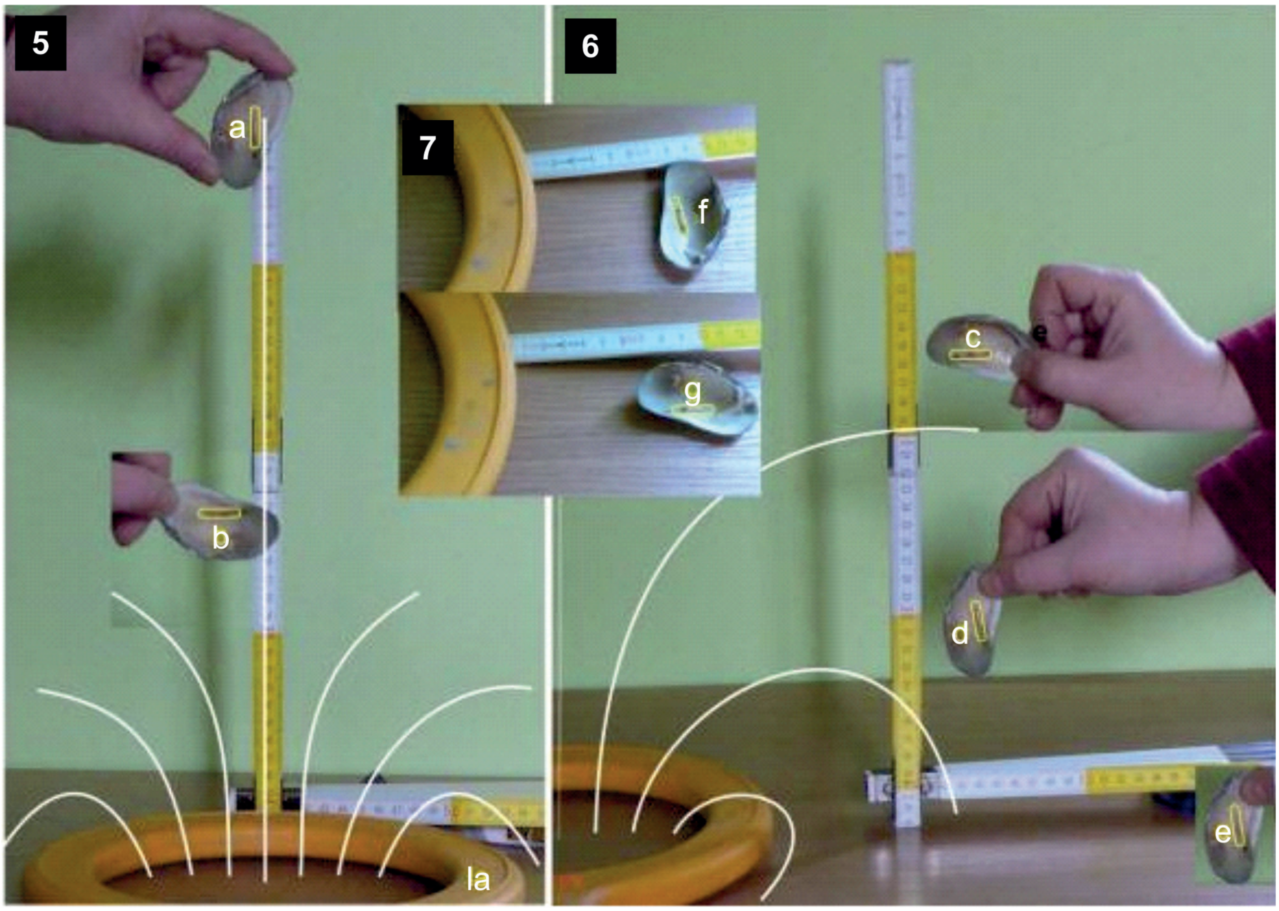
The PIT tags were inserted into the shell using preloaded needles and a special "gun" implanter, which releases the tag from the needle after pressing the trigger. The tags were implanted between the inner shell layer and the mantle: the shell valves were opened forming a chink wide enough (3–5 mm) to manipulate the gun needle. The needle was gently inserted, ca. 1–2 cm deep into the shell along its lower edge and along the longer axis of the mussels' body, between the mantle and the shell, and then the tag was released from the needle.



Figs 1–4. PIT tag attached to the shell of *U. crassus*, found empty in 2015, in the sediment of the bank of the San River: 1 – relative arrangement of the basic elements (ca – coil antenna, f – ferrite core, IC – integrated circuit, s – silicone layer edge), 2 – PIT tag incorporated in the inner shell layer, 3 – close-up view of the thickness and structure of nacre layer, partially removed from the glass of the transponder, 4 – dimensions of the buttress built at the PIT tag end in relation to the elevation

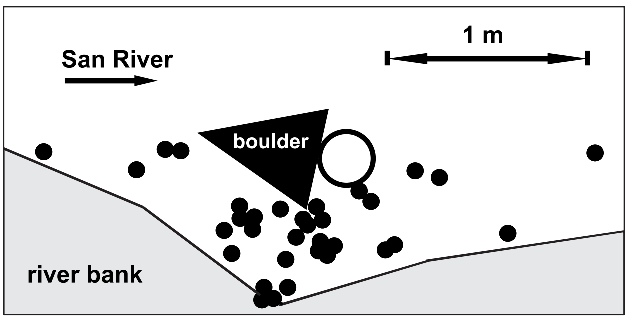
FIELD PROTOCOL

The study was conducted between 2012 and 2015 in the San River near the village Żurawin, S. Poland (49°13'06.2"N, 22°43'05.0"E, 554 m a.s.l.). On April 14th, 2012, 100 individuals of *U. crassus* were marked; only fully grown individuals (over 6 years of life, size over 40 mm) were included in the study. They were collected from the bank of the channel, in one site, taken out of the water for marking, and placed again in a new spot in the channel near a characteristic piece of rock (Fig. 8, black triangle) ca. 1 m from their original site. Their location was inspected again on May 14th, 2012, May 14th, 2013 and December 8th, 2015. On May 14th, 2012, the positions of the mussels in relation to the characteristic piece of rock were mapped precisely and markers were fixed at the bank. At the last control, I inspected the fine sediment bank by hand at a distance of over 30 m from the point of release, checking each found mus-



Figs 5–7. Activating distances of the PIT tag in relation to the distance to the large inducing antenna and the lines of its electromagnetic field (white lines): 5 – activation of PIT tag in the centre of inducing coil antenna (a – PIT tag oriented along the main lines of electromagnetic field, b – PIT tag oriented perpendicularly to the lines – the distance of activation decreases significantly, la – loop antenna), 6 – activation of PIT tag outside the inducing coil antenna (c – despite the horizontal position of the tag, outside the large coil, it conforms to the lines and is activated at a significant distance, d – position of the tag changed, but because the tag orientation conforms to the lines the distance of activation remains large, e – at the plane of the loop the effective position of the tag is vertical, conforming to the field lines), 7 – activation of the tag in the horizontal plane of the inducing antenna (f, g – horizontal positions of both the PIT tags do not conform to the lines which are vertical, thus the distance of activation is significantly reduced in comparison to (e))

sel for the presence of PIT tag. Then I checked the fine sediment bank again with the large inducing antenna, and then I surveyed the stony bottom in the same way ca. 5 m from the bank. Obviously, the hand check of the stony bottom was inefficient and all of the individuals found were detected by the inducing antenna as well. Five tagged individuals were collected and dissected to check the effect of the tag on the nacre secretion.



Another sample of *U. crassus* was marked on April 14th, 2016, when 64 individuals were caught in the Biała River, near Lubaszowa, S. Poland (49°51’40.3”N, 21°02’05.3”E, 223 m a.s.l.). They were both PIT-tagged and marked with alphanumerical code written on the shell with water-hardening glue. They were put in their original site within the channel and inspected again on April 27th, May 2nd, May 10th, May 17th, and May 23rd of 2016.

Fig. 8. Out of the 100 individuals marked with transponders (on April 14th, 2012) and left together in one place (large open circle) behind the boulder (black triangle), 39 were detected (black dots) one month later; all of them had moved toward the bank

RESULTS

Out of the 100 individuals marked with PIT tags in April 2012, 39 were found on May 14th, one month later (Fig. 8), whereas two months later only 13 were re-captured. Of those, seven had been found already on May 14th, whereas the rest had not. They dispersal distance was small, not exceeding 3 m.

On December 18th, 2015, 18 live individuals were re-captured, 12 of them in the vertical, fine sediment slope of the bank. Two dead individuals, found as empty shells burrowed within the fine sediment, had their transponders fixed in the nacre. Fifteen signals were received from the rocky bottom. Based on these, six individuals were found alive, while nine signals were coming from the rocky bottom and, despite efforts, the individuals carrying these devices were not found. Probably a large piece of rock (10 × 30 × 30 cm), when situated between the mussel and the large inducing antenna, could prevent induction of the PIT tag.

Five individuals from the San River were dissected in order to check the position and fixing of the PIT tag. In four of them, the tag was covered by the nacre (Figs 5–7), similarly as in the shells left after the two dead individuals. However, in the empty shells, the nacre layer was damaged at the outermost surface (Fig. 1). In all cases the nacre layer was very thin, of less than 100 μm (Fig. 2). Closer to the shell the nacre was several times thicker, usually covering the tag base with a thick layer (Figs 3, 4, 9–11), adjacent to the inner part of the shell. The transponder was frequently adjoined by a kind of buttress (yellow arrows in Figs 10 and 11; see also Fig. 2 for its structure). They were formed by a much thicker layer of nacre, up to ca. 1 mm. No damage to the soft tissues of the dissected mussels was noticed.

The frequency of tag rejection was estimated during the marking in Lubaszowa in the Biała River, where the site was surveyed at shorter intervals. Among the 64 doubly-marked (painted code and PIT tag) individuals that were later followed every week, 20 rejected the PIT tags on the next survey (31%). They were re-tagged, and out of these 13 rejected the tags again, with the remainder retaining them. In 44 individuals the tags were found on all subsequent controls. After ca. 2 weeks, no tag rejection occurred and during subsequent controls it was observed that the mantle adhered to the shell again but the tags were still moveable.

During the study two individuals died from natural causes (reproduced before death, functioning normally). Their death enabled tag inspection ca. two months after implantation (April 14th to June 16th). The examination revealed that the tag was fixed to the shell with a "gluing" nacre layer, whereas the glass bulb was covered with scattered or adjoining nacre granules (Fig. 9).

Figs 9–11. Transponders fixed in the nacre within the inner layer of the shell of *U. crassus*: 9 – transponder only two months after implantation: fixed at its base adjacent to the shell, on the glass bulb of the PIT tag, are the initial grains of nacre, 10 – transponder after 2 years fixed in the nacre, viewed from above (adjacent to the mussels' flesh) it is covered only with a thin layer of nacre, 11 – transponder almost completely embedded in the nacre, viewed from above. Black arrows indicate the "buttresses" probably additionally fixing the tag

DISCUSSION

The implantation of preloaded PIT tags was very quick; it took no longer than half a minute to handle the mussel and to insert the tag. Obviously, successful implantation needs some training (KURTH et al. 2007), and inappropriate insertion could cause stress or even death if the animal was injured during the procedure (e.g. by needle) or stayed out of the water for too long. In their experiments WILSON et al. (2011) found that inactive mussels were by ca. 10% lighter, which in my opinion indicated that their shells had become filled with air during the prolonged procedure of tag attachment, with a smaller amount of water enclosed in the shell (hence the decrease in weight). This made the animals' delicate tissues dry and impaired their life functions. They mussels also showed reduced activity due to more complicated and prolonged handling and due to the effect of fixing an external PIT tag (a similar line of reasoning, although with different arguments, was presented by HUA et al. 2015).

The most serious problem encountered during implantation of PIT tags is the initial tag rejection. After implantation, the mantle is diverted from the shell. The tag is loosely placed in the resulting space. Until the mantle closely adheres to the shell again, the tag can drop out, which might be caused even by the first movement of the foot outside the shell (rejected tags can be found in a container with tagged mussels before their release into the river). The rejection implies that the individual should be also marked externally, even if the mark is temporal, to be sure about which individual has lost its tag.

From the point of view of short-term individual marking, it makes no difference whether tags are held inside the shell by the mantle or nacre-cemented; it only becomes important in case the mussel dies, its soft tissue decomposes and the tag not fixed in nacre can fall out. This implies that marking has to be completed well before the planned study (1–2 months), to ensure that the tags are well placed and will not be rejected. Repeated implantation usually fails, probably due to the separation of the mantle from the shell over too large an area as a result of repeated operation. In this study no mortality or impairment of life functions (e.g. loose shell closing and change in body colour) were observed among the marked mussels.

The PIT tag technology failed as a method of telemetry and as a way of locating individuals in difficult field conditions. After one month, I failed to find over two-thirds of the marked mussels. The re-captured individuals had not dispersed much, and so it was unlikely that those not found escaped from the range of the study. They did not die because then it would have been easy to find their empty shells in the sediments; moreover, no large floods occurred, which could have washed them away, and there were no traces of predation (ZAJĄC 2014). These conclusions are confirmed by the fact that some of those not found one month after release were found on subsequent controls (similar results in ZAJĄC & ZAJĄC 2011). It also indicates that the PIT tags were not destroyed physically and thus undetectable. An additional confusing factor comes from the tag rejection. Because the tag is heavier than water, it sinks to the bottom and can be detected as a "false" signal of the marked mussel. Due to its size it is very difficult to find, which leads to a high level of confusion during subsequent surveys.

Tags are not precisely detected in difficult habitats such as montane rivers. The tags were not detectable with the standard-inducing antenna when the whole PIT tag was even slightly immersed in water. Therefore, the stronger-inducing antenna had to be used. Because the signal in the water is much weaker, the PIT tags were detected only beneath the centre of the antenna loop or just at the edge, at very small distances. The strength of the antenna signal could be reduced using the fader, a part of the antenna equipment. Fading the signal allowed for reducing the detectability practically to the centre of the large antenna loop; however, in such a situation it was unlikely to detect individuals whose tags were probably not perpendicular to the loop plane or those that were hidden deeper in the substratum. In such cases, the loop's position can be adjusted in relation to the bottom to detect individuals with an incorrect tag position (see Figs 5–7). Increasing the strength of the antenna is inefficient; since the signal comes from a large area, it is very difficult to locate the marked animal precisely, and the standard equipment does not measure the signal's strength but only reads the code as "all or nothing". When the mussels are stuck in the vertical bank of the channel built of fine sediment, their position is, as a rule, perpendicular to the bank and thus also to the loop of the large-inducing antenna. In such a case the detectability is 100%.

It is recommended – even in habitats that are easy to search – to implant the transponders in all the individuals in exactly the same manner (i.e. position in relation to the shell) in a given habitat, so that the orientation of the large antenna loop is properly set in relation to the predicted position of the mussel during the survey. Because the plane of the siphons is usually perpendicular to the long axis of the shell, and when the siphons in a given habitat are usually kept parallel to the bottom (regardless of whether it is a flat bottom of the channel or a vertical slope of the bank), the best orientation of the tag would be the position along the long axis of the shell (Figs 5–7). PIT tags vary in size, which also influences the strength of the signal. Mussel marking studies have been carried out on PIT tags ranging in length from 9 mm (GOUGH et al. 2012) to 32 mm (FISCHER et al. 2012), but currently PIT tags as small as 7 mm in length are available. HUA et al. (2015) used 12 mm tags for external marking of mussels as small as 20 mm.

PIT tags have already been recognised by KURTH et al. (2007) as a useful tool enabling re-capturing of tagged mussels. These authors preferred using tags by making an internal incision rather than by surface fixing with cement, because the external fixing was more time-consuming and less durable, especially over longer periods (several months), which needed occasional re-cementation. This technique was also advocated by FISCHER et al. (2012) for detecting freshwater mussels on large spatial scales, using large devices attached to boats. Such devices, being more powerful, could be used in deeper waters due to the larger carrying capacity of a boat. HUA et al. (2015) marked young captive-bred mussels with PIT tags on a large scale to check the effectiveness of their release into the wild – to restore the population of a threatened species – and found very high survival rates. At present, most researchers prefer the use of PIT tags glued on to the external part of shell, especially in short-term studies (M. LOPES-LIMA, pers. comm.), to avoid problems with tag rejection; however, the gluing is non-permanent and can be problematic on stony substrata.

CONCLUSION

PIT tag technology is a suitable method which ensures durability, as the recovered PIT tags were found to be immersed in the nacre and fixed very firmly, and still operating after three years. The technology can be used successfully for locating individuals in captivity or in the wild, but only on the kind of bottom which is easy to search (e.g. flat sandy bottom in small streams, where the position of the PIT tag micro antenna is similar in all individuals and can be predicted; see GOUGH et al. 2012, also HAMILTON & CONNEL 2009 for sea sand habitats). Internal tag implantation seems to be a durable method in aquatic habitats and seems more durable than external marking. However, for long-term marking this method requires tag insertion early enough to control possible tag rejection. The additional function of locating with an inducing antenna can sometimes be useful in advantageous circumstances or, regardless of the effort needed, in special, emergency situations, for example high turbidity (NEWTON et al. 2015) or algal growth, when PIT tags technology allows for locating marked mussels regardless of the visibility. Therefore, it can be concluded that PIT tag technology is a method more suitable for ID marking and less suitable for detecting and/or locating of the marked mussels.

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